SHOUT
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IT

BIOLOGICAL BULLETIN

OF THE

Marine Biological Laboratory

WOODS HOLE, MASS.

Editorial Staff

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VOLUME XLVII.

WOODS HOLE, MASS.

JULY TO DECEMBER, 1924

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BIOLOGICAL BULLETIN

THE MARINE BIOLOGICAL LABORATORY.

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I. TRUSTEES (AS OF AUGUST 14, 1923).

EX OFFICIO.

FRANK R. LILLIE, *Director*, The University of Chicago.

GILMAN A. DREW, *Assistant Director*, Marine Biological Laboratory.

WILLIAM H. DUNBAR, *Treasurer*, 161 Devonshire Street, Boston, Mass.[1]

GARY N. CALKINS, *Clerk of the Corporation and Secretary of the Board of Trustees* Columbia University.

EMERITUS.

CORNELIA M. CLAPP, Mount Holyoke College.

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[1] Mr. Dunbar resigned as Treasurer, January 7, 1924, and Mr. Lawrason Riggs, Jr., 25 Broad St., New York City, was elected by the Trustees in his stead.

TO SERVE UNTIL 1927.

H. C. Bumpus, Brown University.
W. C. Curtis, University of Missouri.
Jacques Loeb,[1] The Rockefeller Institute for Medical Research.
George T. Moore, Missouri Botanical Garden, St. Louis.
W. J. V. Osterhout, Harvard University.
J. R. Schramm, Cornell University and National Research Council.
William M. Wheeler, Bussey Institution, Harvard University.
L. L. Woodruff, Yale University.

TO SERVE UNTIL 1926.

E. G. Conklin, Princeton University.
Otto C. Glaser, Amherst College.
Ross G. Harrison, Yale University.
H. S. Jennings, Johns Hopkins University.
F. P. Knowlton, Syracuse University.
M. M. Metcalf, Oberlin, Ohio.
William Patten, Dartmouth College.
W. B. Scott, Princeton University.

TO SERVE UNTIL 1925.

C. R. Crane, New York City, *President of the Corporation.*
I. F. Lewis, University of Virginia.
R. S. Lillie, Nela Research Laboratories.
E. P. Lyon, University of Minnesota.
C. E. McClung, University of Pennsylvania.
T. H. Morgan, Columbia University.
D. H. Tennent, Bryn Mawr College.
E. B. Wilson, Columbia University.

TO SERVE UNTIL 1924.

H. H. Donaldson, Wistar Institute of Anatomy and Biology.
W. E. Garrey, Tulane University.
Caswell Grave, Washington University.
M. J. Greenman, Wistar Institute of Anatomy and Biology.
A. P. Mathews, The University of Cincinnati.
G. H. Parker, Harvard University.
C. R. Stockard, Cornell University Medical College.

Executive Committee of the Board of Trustees.

Frank R. Lillie, *Ex. off. Chairman.*
Gilman A. Drew, *Ex. off.*

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[1] Deceased.

E. G. CONKLIN, to serve until 1926.
W. E. GARREY, to serve until 1925, *Secretary.*
H. H. DONALDSON, to serve until 1924.

THE LIBRARY COMMITTEE.

C. E. MCCLUNG, Chairman, University of Pennsylvania.
M. H. JACOBS, University of Pennsylvania.
E. E. JUST, Howard University.
M. M. METCALF, Oberlin, Ohio.
J. R. SCHRAMM, Cornell University and National Research Council.

II. ACT OF INCORPORATION.

No. 3170

COMMONWEALTH OF MASSACHUSETTS.

Be It Known, That whereas Alpheus Hyatt, William Sanford Stevens, William T. Sedgwick, Edward G. Gardiner, Susan Minns, Charles Sedgwick Minot, Samuel Wells, William G. Farlow, Anna D. Phillips and B. H. Van Vleck have associated themselves with the intention of forming a Corporation under the name of the Marine Biological Laboratory, for the purpose of establishing and maintaining a laboratory or station for scientific study and investigation, and a school for instruction in biology and natural history, and have complied with the provisions of the statutes of this Commonwealth in such case made and provided, as appears from the certificate of the President, Treasurer, and Trustees of said Corporation, duly approved by the Commissioner of Corporations, and recorded in this office;

Now, therefore, I, HENRY B. PIERCE, Secretary of the Commonwealth of Massachusetts, *do hereby certify* that said A. Hyatt, W. S. Stevens, W. T. Sedgwick, E. G. Gardiner, S. Minns, C. S. Minot, S. Wells, W. G. Farlow, A. D. Phillips, and B. H. Van Vleck, their associates and successors, are legally organized and established as, and are hereby made, an existing Corporation, under the name of the MARINE BIOLOGICAL LABORATORY, with the powers, rights, and privileges, and subject to the limitations, duties, and restrictions, which by law appertain thereto.

Witness my official signature hereunto subscribed, and the seal of the Commonwealth of Massachusetts hereunto affixed, this twentieth day of March, in the year of our Lord One Thousand, Eight Hundred and Eighty-Eight.

[SEAL] HENRY B. PIERCE,
Secretary of the Commonwealth.

III. BY-LAWS OF THE CORPORATION OF THE MARINE BIOLOGICAL LABORATORY.

I. The annual meeting of the members shall be held on the second Tuesday in August, at the Laboratory, in Woods Hole, Mass., at 12 o'clock noon, in each year, and at such meeting the members shall choose by ballot a Treasurer and a Clerk, who shall be, *ex officio*, members of the Board of Trustees, and Trustees as hereinafter provided. At the annual meeting to be held in 1897, not more than twenty-four Trustees shall be chosen, who shall be divided into four classes, to serve one, two, three, and four years, respectively, and thereafter not more than eight Trustees shall be chosen annually for the term of four years. These officers shall hold their respective offices until others are chosen and qualified in their stead. The Director and Assistant Director, who shall be chosen by the Trustees, shall also be Trustees, *ex officio*.

II. Special meetings of the members may be called by the Trustees to be held in Boston or in Woods Hole at such time and place as may be designated.

III. The Clerk shall give notice of meetings of the members by publication in some daily newspaper published in Boston at least fifteen days before such meeting, and in case of a special meeting the notice shall state the purpose for which it is called.

IV. Twenty-five members shall constitute a quorum at any meeting.

V. The Trustees shall have the control and management of the affairs of the Corporation; they shall present a report of its condition at every annual meeting they shall elect one of their number President and may choose such other officers and agents as they may think best; they may fix the compensation and define the duties of all the officers and agents; and may remove them, or any of them, except those chosen by the members, at any time; they may fill vacancies occurring in any manner in their own number or in any of the offices. They shall from time to time elect members to the Corporation upon such terms and conditions as they may think best.

VI. Meetings of the Trustees shall be called by the President, or by any two Trustees, and the Secretary shall give notice thereof by written or printed notice sent to each Trustee by mail, postpaid. Seven Trustees shall constitute a quorum for the transaction of business. The Board of Trustees shall have power to choose an Executive Committee from their own number, and to delegate to such Committee such of their own powers as they may deem expedient.

VII. The President shall annually appoint two Trustees, who shall constitute a committee of finance, to examine from time to time the books and accounts of the Treasurer, and to audit his accounts at the close of the year. No investments of the funds of the Corporation shall be made by the Treasurer except approved by the finance committee in writing.

VIII. The consent of every Trustee shall be necessary to dissolution of the Marine Biological Laboratory. In case of dissolution, the property shall be given to the Boston Society of Natural History, or some similar public institution, on such terms as may then be agreed upon.

IX. These By-laws may be altered at any meeting of the Trustees, provided that the notice of such meeting shall state that an alteration of the By-laws will be acted upon.

X. Any member in good standing may vote at any meeting, either in person or by proxy duly executed.

IV. THE REPORT OF THE TREASURER.[1]

Harvey L. Chase & Company,
84 State Street, Boston.

February 5, 1924.

William H. Dunbar, Esq.,
161 Devonshire Street,
Boston.

Dear Sir: We have completed our audit of the accounts of the Marine Biological Laboratory for the year ended December 31, 1923, and report thereon in the accompanying exhibits and schedules:

Exhibit A—Balance-Sheet as of December 21, 1923.
Exhibit B—Income-and-Expense for the Year ended December 31, 1923.
Schedule I—Investments (Book Values).
Schedule II—Cash Receipts and Disbursements on Account of Funds.
Schedule III—Land, Buildings, and Equipment.
Schedule IV—Supply Department Income-and-Expense Account for the Year ended December 31, 1923.

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[1] Only a part of the audit is included in this report. The complete audit is on file at the Laboratory, and may be examined by any member.

Schedule V—Analysis of Plant Costs and Fire Insurance in Force.

We certify that, subject to the comments herewith, the balance-sheet and income-and expense statement shown in Exhibits A and B are in accordance with the books and correct, to the best of our knowledge and belief.

Very respectfully,
(Sgd.) HARVEY L. CHASE & COMPANY,
Certified Public Accountants.

MARINE BIOLOGICAL LABORATORY BALANCE SHEET, DECEMBER 31, 1923.

Assets.

Cash:		
In bank	$ 1,332.49	
Petty Cash Fund	200.00	$ 1,532.49
Accounts receivable		16,239.42
Inventories:		
Supply department	22,459.15	
BIOLOGICAL BULLETIN	3,528.37	25,987.52
Investments:		
Securities (Schedule I)	12,151.12	
Less Loan	1,100.00	
	11,051.12	
Cash (Schedule II)	1,176.84	12,227.96
Stock in General Biological Supply House, Inc.		12,700.00
Gansett property account	11,531.27	
Less mortgage	8,182.01	3,349.26
Educational Plant:		
Land	101,006.14	
Buildings	226,452.28	
Equipment	103,144.80	
	430,603.22	
Less reserve for depreciation	60,160.36	370,442.86
Prepaid insurance		2,774.27
Items in suspense		250.91
		$445,504.69

Liabilities.

Accounts payable	$ 966.74
Notes payable	5,000.00
Trust funds	12,227.96

Balancing account:		
Balance, January 1, 1923	$410,671.88	
Add:		
Special Donation received from C. R. Crane for final payment on purchase of Fish Estate (Kidder House)	15,000.00	
Balance of Income for Year (Exhibit B)	1,638.11	427,309.99
		$445,504.69

MARINE BIOLOGICAL LABORATORY, INCOME AND EXPENSES FOR YEAR ENDED DEC. 31, 1923.

	Expenses	Income	Loss	Gain
Administrative expenses	$ 9,880.21	$	$ 9,880.21	$
Bar Neck Property expense	375.00		375.00	
BIOLOGICAL BULLETIN and annual dues	3,602.61	3,798.68		196.07
BIOLOGICAL BULLETIN adjustment of expenses for 1922	448.48		448.48	
Carpenter department	1,315.23	30.81	1,284.42	
Chemical department	1,937.29		1,937.29	
Evening lectures	110.20		110.20	
Dormitories	3,473.48	3,229.73	243.75	
Instruction	7,144.26	10,862.50		3,718.24
Interest on notes payable	674.13		674.13	
Janitor's house expenses	7.69		7.69	
Library department	1,909.61		1,909.61	
Maintenance, buildings and grounds	6,474.87		6,474.87	
Mess	26,000.48	28,157.30		2,156.82
New laboratory	4,762.95		4,762.95	
Newman cottage	129.84	150.00		20.16
Pumping station	512.59		512.59	
Research department	3,125.77	6,825.00		3,699.23
Supply department (see Schedule IV)	50,210.33	48,461.17	1,749.16	
Sundry expense and income	921.52	13,457.74		12,536.22
Truck	607.77		607.77	
Total current expenses	$123,624.31		$30,978.12	
Total current income	$114,972.93	$114,972.93	$22,326.74	$22,326.74
Excess of expenses	$ 8,651.38		$ 8,651.38	
Reserve for depreciation	9,109.84			
Bad accounts written off	600.67			
	$ 18,361.89			
Donations for expenses, Friendship Fund, Inc.	20,000.00			
Balance to Exhibit A	$ 1,638.11			

MARINE BIOLOGICAL LABORATORY INVESTMENTS, DECEMBER 31, 1923.

Reserve Fund.

Cash on hand	$ 782.93	
*$3,000.00 American Telephone & Telegraph Company, 4's	2,921.25	
500.00 Western Telephone & Telegraph Company, 5's	496.88	
* 6 shares American Smelting & Refining Company, Preferred	732.00	
8 shares General Electric Company	907.25	
8 shares General Electric Company Special (par $10.00) received as a stock dividend.		
14 shares United Shoe Machinery Corporation, Preferred.	393.75	
5 shares Massachusetts Gas Companies, Preferred	444.63	
	$6,678.69	
Items marked * are held as collateral on loan of	1,100.00	$ 5,578.69

Library Fund.

Cash on hand	$ 20.13	
$300.00 U. S. Liberty Loan, First 4 1/4's	300.00	
4/5 of $1,000.00 American Telephone & Telegraph Company, 4's	779.00	
3 shares American Telephone & Telegraph Company	362.38	
3 shares General Electric Company	346.80	
3 shares General Electric Company Special (par $10.00) received as stock dividend		
5 shares United Shoe Machinery Corporation, Preferred	140.63	
3 shares Massachusetts Gas Companies, Preferred	269.38	
*1 shares American Smelting & Refining Company, Preferred	122.00	2,340.32

Lucretia Crocker Fund.

Cash on hand	$ 373.78	
$300.00 U. S. Liberty Loan, 1st 4 1/4's	300.00	
1/5 of $1,000.00 American Telephone & Telegraph Company, 4's	194.75	
18 shares Vermont & Massachusetts Railroad Company	2,416.50	
3 shares General Electric Company	349.55	
3 shares General Electric Company Special (par $10.00) received as stock dividend.		
1 shares Boston Elevated Second Preferred	133.00	
1 share American Telephone & Telegraph Company	120.79	
4 share Boston Consolidated Gas Company, Preferred	420.58	4,308.95
Total, Exhibit A		$12,227.96

V. REPORT OF THE ASSISTANT LIBRARIAN.

The increase in the rate of growth of the Library since 1920 is encouraging considering the increasing cost of books during this time and the very slight increase of our 1923 budget over that of 1920. In these four years the number of accessions of volumes has been, in sequence, 408, 403, 473 and 562; of reprints, 387, 427, 434, and about 800 for 1923, but only 194 of these will show in our records of the year because the work of recording and cataloguing the remainder will be delayed until 1924. Moreover, the numbers of volumes accessioned in the years 1920 and 1921 were greatly increased by the binding of back sets of German serial publications that were purchased from a special fund for this purpose and not from the years' appropriations. The reprints which are a most useful part of the Library involve a great deal of work in their cataloguing and care, and their increase, which we hope will continue, has made a great jump this past year. Also there should be mentioned an accumulation of about 1000 or more of these, dating far back, that were sorted out alphabetically last year from the mass of miscellaneous printed matter that was accumulated before 1919. These will have to be accessioned as soon as adequate assistance in the Library staff make it possible.

Of the volumes acquired by the Library in 1923, 328 were recorded from binding, 126 of these being exchanges and the remainder our paid subscriptions, except 8 that were from binding the American Museum of Natural History loans; 151 of the accessioned volumes were direct from purchase, 65 by gift, and 20 from our exchanges.

There are now in the Library about 11,698 volumes and 9,587 reprints.

The Library has received during the past year current serial publications to the number of 281. We have paid our subscriptions to 93 of these; sixty four (64) subscriptions of the Biological Bulletin have been used by the Library in exchange for 87 of these currently received serials; we have received 80 as gifts; and 21 through the American Museum of Natural History loan.

The new subscriptions undertaken in 1923, on our paid list, were: the *Journal of Anatomy*; *Annual Report of the Progress of Chemistry; Zeitschrift für Botanik;* and the *Botanische Jahrbucher für Systematik, Pflanzengeschichte und Pflanzengeographie.* The new exchanges were: the *Philosophical Transactions* and the *Proceedings of the Royal Society of London, Series B; British Journal of Experimental Biology; Tydschrift der Nederlandisch Dierkundige Vereeniging; Bolletin and Memorias de la Real Sociedad Espanola de Historia natural; Bulletin du Museum National d'Histoire Naturelle;* and the *Tokyo Botanical Magazine.* Our major purchases in books have been Aberhalden's: "Handbuch der biologischen Arbeitsmethoden, all so far issued; Brandt and Apstein's "Nordisches Plankton," save the three numbers on the Protozoa which we already had; and the three volumes of Mellor's "Inorganic Chemistry" then issued.

The binding was extraordinarily heavy this year on account of several unbound sets that have been secured by exchange during the past two years. But we have brought our binding now to a satisfactory relation to current receipts, whereas since 1919 we had been in arrears on account of back volumes received after the delay caused by the war.

The appropriation for the use of the Library for 1923, aside from salaries, was two thousand dollars. Properly there should be added to this the value of sixty-four (64) yearly subscriptions of the Biological Bulletin that have been used by the Library for exchange purposes. The two thousand was apportioned as follows: current subscriptions, $750; books, $350; Binding, $750; and supplies and express, $150. Though these apportionments were not strictly adhered to, the total sum spent, as shown on our Library Account Book, is $1,999.58. The items that ran over our estimate, being subscriptions and books, both due to the very sudden advance in German prices on the change of their method of billing from Marks to Dollars.

In order to keep up our present subscriptions we shall have to increase our subscription apportionment to the sum of $1,000.00, and even this will not allow us to pay for current subscriptions for the year ahead, as we should like in those cases where we can get accurate bills for these in December. About $200.00 more would be needed in order to start January 1925 with no bills on hand.

We are making every effort to secure some of the German serials that we now subscribe for, by exchange of the BIOLOGICAL BULLETIN. And our German dealer is coöperating with us in this, to the extent of encouraging us to approach the publishers and editors of these publications with this offer in mind. If we cannot cut down our subscription expense in this way, we shall have to receive a material increase for this item for the year 1925 in order to continue everything on our list. The need of systematically filling gaps in our sets and of securing back sets of new subscriptions that we take up from time to time, has been before us for so long and efforts made to get these by haphazard methods has proved so unsatisfactory, that we are making a special apportionment in our budget hereafter for this purpose. Another need is for a yearly sum to pay for assistance in the Library. The care of steadily increasing amount of material, received each year since 1919, should not be allowed to hamper the efforts of the Assistant Librarian to go on with the work of expansion of the Library.

In November of this year a printed circular was sent to each member of the Corporation of the Laboratory, soliciting his personal interest in the Library, and asking that he present to the Library any numbers or volumes of the Biological Bulletin that he find available. A fine gift of a complete set from volume 3, no. 4 to volume 45 complete, was presented by Dr. Rankin of Princeton and the value of this has been credited to the Library to go on our budget for back sets, a special item just referred to. From Miss Caroline Thompson's Library at Wellesley we received, among other numbers, two that will enable us to complete volumes of the BIOLOGICAL BULLETIN that are out of print. Other numbers have been promised and we hope that the response to our appeal specifically for the Biological Bulletins, and in general for an active interest in and coöperation with the Library is not yet ended.

PRISCILLA B. MONTGOMERY,
Assistant Librarian.

VI. THE DIRECTOR'S REPORT.

To the Trustees of the Marine Biological Laboratory.

Gentlemen: I beg to submit herewith a report of the thirty-sixth session of the Marine Biological Laboratory for the year 1923. On account of the completion of our program for building and endowment in January, 1924, it seems desirable to include the events of that month also, so far as they relate to that project.

1. *Attendance.*—The attendance of students in courses was 146 and of investigators 176, making a total of 322 persons, representing 107 institutions. Applications for working places were again considerably greater than our accommodations, especially in the case of places for students in courses. Our policy of instruction has been guided for many years by the desire to contribute to the production of investigators. The results might be judged by the extent to which students in the courses of instruction have acquired the spirit of research by their work and contacts at Woods Hole. Unfortunately no quantitative measurements of such a sort can be directly made; however, we have strong reasons to believe from our own experience, as members of the Marine Biological Laboratory and professors in many colleges and universities, that many of our students acquire a better understanding of the spirit, methods and standards of research at Woods Hole, and that their ambition is stimulated. A measureable evidence of the influence of our classes is furnished by the number of former members who have published research work. An examination of this question was made by Dr. Drew, who took the list of all the members of the classes in Inevertebrate Zoölogy, in Embryology, in Physiology and in Botany between the years 1908 and 1917, and submitted each list to ten investigators to check the names of those known to have published research work. Of the 350 members of the classes in Invertebrate Zoölogy during this decade, 63, or 18 percent, were checked; of the 197 in Embryology, 65, or 33 percent, were checked; of the 93 in Physiology 49, or 53 percent, were checked; of the 111 in Botany 43, or 39 percent, were checked. It may be of additional interest to note that for the years 1908–1912 (chosen to give an adequate perspective) 12 percent of those registered in Invertebrate Zoölogy have been

listed in "American Men of Science," 24 percent of those registered in Embryology, 20 percent of those registered in Physiology, and 26 percent of those registered in Botany. The results certainly show that a large percentage of the students taking courses have professional interests, whether acquired as a result of attending the Laboratory or not. In more recent years the demand for the courses has been steadily increasing, and while it is too early to say yet from the evidences of publication, it is nevertheless our strong impression that the professional interest has been increasing.

With the present excess of applicants for the courses, it seems possible both to stress more the intent of the courses as training for research, and also to devise better methods of selection, with this aim in view, from the applicants who present themselves. It was accordingly decided by the Executive Committee on the recommendation of the Board, to set definite numerical limits to each of the classes, and to devise a form of application for admission better suited to bring out the information necessary for selection on this principle. The new form of application is printed on pp. 36–38.

The number of subscribing and coöperating institutions increased from 61 in 1922 to 71 in 1923. Of the total amount of $11,600 received from these institutions $5,425 was subscribed for students' tables and $6,175 for research accommodations. Hitherto these institutions have enjoyed priority rights of admission for their students up to a certain number from each. This has involved certain difficulties of administration, with reference especially to admission of students from other institutions. It was accordingly decided by the Executive Committee to do away with right of priority for students' places, and to accept applicants solely on their merits; this action is without prejudice to the rights of such institutions in their research accommodations. We hope and believe that the necessity of this decision, which has been gradually approached by successive limitations of priority rights to students' places, will be recognized by the institutions concerned. The Marine Biological Laboratory belongs to all American institutions in which the spirit of investigation is living, and equal opportunity for admission of students should be open to all.

2. *Library.*—The resignation of the Librarian, Dr. R. P. Bigelow, was presented during the year and accepted with regret by the Trustees, who expressed to him their appreciation and that of the Laboratory for his efficient and valuable services. Dr. Bigelow brought to the aid of the Laboratory in his capacity of Librarian a rich fund of experience acquired as Librarian of the Massachusetts Institute of Technology. The organization of our library owes much to his experience. On recommendation of the Library Committee it was voted to request the Assistant Librarian to conduct the affairs of the library in consultation with the Library Committee pending the appointment of a Librarian.

Mrs. Montgomery's very interesting report as assistant librarian on the growth of the library in 1923 is printed on page 9. The recommendations of that report can certainly be carried out with the increased space to be provided in the new building and the increased income proposed to be devoted to library purposes. It should be our aim under the new conditions to undertake the large task of building up the best possible working library for biological research as rapidly as can be done. This can be accomplished only if the Library Committee receives the active coöperation of the members of the Laboratory. Special funds should be secured and set aside to enable the library administration to take advantage of all unusual opportunities for purchase of rare sets. The library is a department in which every one can be of assistance, for every addition helps, and a great library can be built up only bit by bit.

3. *The Report of the Treasurer* (See pp. 5–8).—The earned income of the Laboratory during the past five years has been as follows: 1919, $80,307.57; 1920, $89,943.04; 1921, $98,292.38: 1922, $108,686.37; 1923, $114,972.93. The current expenses during the same periods were: 1919, $88,452.64; 1920, $103,097.64; 1921, $109,970.40; 1922, $113,466.83; 1923, $123,624.31. During the same years the annual contributions of $20,000 a year from the Friendship Fund have balanced the account and left a surplus for necessary expansions. The rapid increase of expenses, amounting to about 40 percent, in five years would be alarming if it were not compensated by an equally rapid increase in earnings, approximately 43 percent, in the same

period. The figures show how much the Laboratory stands in constant need of the coöperation of all of its members in avoiding unnecessary requisitions for expenditures, and in securing support from their institutions. Another way in which members of the Laboratory can aid is by patronizing the Supply Department and the General Biological Supply House for their laboratory supplies as much as possible.

During the current year the Laboratory was able to pay off half of its current indebtedness, or $5000. The amount came partly from earnings and partly from the General Biological Supply House in which the Laboratory is a majority stockholder; this corporation has now become firmly established and was able to pay up all of its back dividends.

The Treasurer of the Laboratory for over twenty-three years, Mr. D. Blakely Hoar died on March 8, 1923. An appreciation of his services is given elsewhere in this report, but the Director may be permitted in this place to give his personal testimony to the high-minded, courteous, efficient and friendly way in which Mr. Hoar for so many years served the Laboratory; the reponsibility was always carried with constant and genuine devotion to our interests. After the death of Mr. Hoar, Mr. William H. Dunbar, senior partner of the firm with which Mr. Hoar has been so long connected, consented to accept the office of Treasurer pending the completion of our campaign for building and endowment funds. This generous action was of the greatest service to the Laboratory in a real emergency, and cannot be appraised too highly. On the completion of the campaign, Mr. Dunbar resigned, and Mr. Lawrason Riggs, Jr., of New York City was elected Treasurer by the Board of Trustees at their special meeting January 7, 1924. Mr. Riggs' knowledge of affairs and his long acquaintance with the Laboratory and many of its members qualify him in a preëminent way to administer the increased responsibilities of the Treasurer's office. The Laboratory is deeply indebted to him for his consent to serve.

4. *The Gansett Property.*—The sale of lots in the Gansett tract proceeded rapidly in 1923. By the end of the year the amounts due on sales were sufficient to pay off the remaining mortgage indebtedness amounting to $8,182.01, and to complete the grading of the roads, There remained then unsold eleven lots,

two reserved for laboratory purposes and two undivided parcels. In accordance with a vote of the Executive Committee, it was decided to withdraw the remainder from sale until the summer of 1924 pending the determination of a new policy, which should ensure the interest of the Laboratory in its perpetual control of at least a portion of the tract.

5. *Agassiz Memorial.*—In accordance with plans announced last year exercises were held at the Marine Biological Laboratory on August 13 to commemorate the fiftieth anniversary of the founding of the Anderson School of Natural History on the Island of Penikese in 1873 by Louis Agassiz. This was the last considerable event of his life for he died in December of the same year, sixty-six years of age. The Penikese School was continued in the summer of 1874 by his son, Alexander Agassiz, and members of Louis Agassiz' staff. The Laboratory existed only two years; it was under the direction of the master naturalist only one year, yet Marcou, Agassiz' biographer, calls the founding of the Penikese Laboratory the most extraordinary episode in Agassiz' life; and David Starr Jordan, one of Agassiz' associates in 1873, writes in his autobiography "The Days of a Man." "While Penikese is deserted, the impulse which came from Agassiz' work there still lives and is deeply felt in every field of American Science. For with all due appreciation of the rich streams which in later years have flowed from many quarters, it is still true that the school with most extended influence on scientific teaching in America was held in an old barn on a little off-shore island. It lasted only a few months, and it had virtually but one teacher. When he died it vanished."

This event was accordingly celebrated by a largely attended meeting, at which a bronze tablet was presented in duplicate, the original to be set in a boulder on the hill on Penikese overlooking the site of the former Laboratory and the sea beyond, the replica to be set in an appropriate place in the Woods Hole Laboratory. For permission to set the Penikese tablet we are indebted to the Senate and House of Representatives of the State of Massachusetts which passed an enabling bill, and to Governor Cox who interested himself in its passage and signed it. The tablet at Woods Hole will recall to successive generations of naturalists young and old the services of the Great

Naturalist it commemorates, and will be an incentive to visit the site of his last undertaking.

The tablet bears the following inscription:

In Commemoration
of the
ANDERSON SCHOOL OF NATURAL HISTORY
Established Fifty Years Ago on the
Island of Penikese
by
JEAN LOUIS RODOLPHE AGASSIZ
Born 1807 Died 1873
The Marine Biological Laboratory
the Direct Descendant of the
Penikese School Erects this Tablet
1923

A few days after the meeting a party of naturalists went to Penikese with a mason, and the tablet was set on the highest boulder of the island. The replica has been set opposite the memorial tablet of Professor Whitman in the entrance to the Crane Building.

It was hoped that three members of the staff of the Penikese Laboratory would be present to speak at the exercises: Doctor E. S. Morse, Doctor Burt G. Wilder, and Doctor David Starr Jordan; but of these only Doctor Morse was able to attend. Doctor Wilder sent his greetings, and Doctor Jordan sent an address prepared for the meeting which was read by Dr. Drew.[1]

The Director presided and made the opening remarks; Professor Morse spoke of his experiences on Penikese and his recollections of Agassiz; Dr. Cornelia M. Clapp gave an account of the second session of the Penikese Laboratory which she attended and furnished a roster of the attendance of the first session which had never been published. Dr. H. C. Bumpus spoke of the intermediate history of the Island, and Professor E. G. Conklin traced the origin of the Marine Biological Laboratory from the same fertile source.

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[1] Since published in the *Scientific Monthly.*

6. *Building and Endowment Funds:* Since the report for 1919 the Director's report has referred each year to the need of new buildings and endowment to provide for the development and expansion of our work. Efforts were begun in 1919 to secure the funds, estimated at $1,000,000 needed for these purposes. In the report for 1922 the Director reported the contribution of $500,000 from the Rockefeller Foundation and $100,000 from the Carnegie Corporation towards this project, both gifts being conditioned on the raising of the estimated sum required. In December, 1923, Mr. John D. Rockefeller, Jr., contributed $400,000 through Mr. Raymond B. Fosdick, thus assuring the other contributions. On the completion of this sum the Friendship Fund, established by Mr. Crane, endowed its annual contribution of $20,000 a year by turning over to a trust company for the benefit of the Marine Biological Laboratory bonds of the par value of $405,000 yielding $20,020 a year. The Laboratory thus receives $1,405,000 for its future development, of which $500,000 is subscribed for building and $905,000 for endowment. These gifts were all paid in to the Treasurer in January, 1924.

A further contribution to the cost of the building was made by the Architect, Charles A. Coolidge, by his generous offer to reduce by one per cent. the customary charges of his firm for architects' services. This gift, amounting to over $6,000 culminates, for the present, a long series of services by Mr. Coolidge to the Laboratory, as Trustee from 1901 to 1921, as Architect of the Crane Building, and as expert adviser constantly in all our plans for enlargement before they assumed practical form.

The fortunate outcome has been due not alone to the devoted coöperation of our own membership, constituting in itself a strong body of favorable expert opinion, but also to a larger body of scientific public opinion, and especially to the support of the National Research Council. Back of all this, and furnishing the foundation, lies the continuous and unwearying support, financial and moral, furnished since 1902 by the President of the Board of Trustees, at first personally, and later, as concerns financial support, through the Friendship Fund endowed by him. One of the greatest of our satisfactions must be the justifying of Mr. Crane's confidence in the future of our Institution.

At a special meeting of the Board of Trustees held on January 7, 1924, a Committee of three members was appointed to draw up official acknowledgments expressing appreciation of the Trustees to the donors of the funds and to the National Research Council. The Trustees also authorized the construction of the new building at Woods Hole in general accord with the plans submitted by Dr. Drew to the meeting and within the limits of the gifts and pledges made for the purpose, and appointed the Director, Assistant Director and Treasurer as a Building Committee to supervise the construction. The Director and Treasurer were moreover especially authorized to sign the contracts relating to the building. Provision was also made for Trusteeship of funds given for endowment.

In the Director's Report for 1921 the question of endowment is referred to. It is there stated that we were not asking for a large endowment, but only for enough so that the operation of the new addition should not be unduly burdensome. It should be realized by all of our members that the income from the new endowment is not sufficient to release us from those customary sources of support that are won by our own coöperation and endeavors; indeed the administration of the new laboratory and the enlarged library may easily put upon us the need for greater efforts than we have made before. The endowment merely provides a more assured financial foundation upon which to build. It is an argument for increase of efforts, not for decrease—an incentive to stronger morâle in our membership, not an excuse for its weakening.

The new building and endowment came to us without any conditions attached. The Director, however, with the concurrence of the Board of Trustees, requested the donors to place the endowment funds in trust. The reasons for this request are given in the Director's Report for 1921 as follows: "The membership of our Corporation and Board of Trustees is composed almost entirely of scientific men; such a body is not usually considered well qualified to deal with matters of investment connected with relatively large endowments. On the other hand, to create another body, as an integral part of the Laboratory, that would have control of finances, would inevitably weaken the control of the scientific men over the policies of the Laboratory in general. Our Board of Trustees for these reasons adopted the policy that any endowment that should be created in connection with the present undertaking should be held in trust for the Laboratory by an outside body, which should pay in the income to the Laboratory for operating expenses."

The Trust Deed is printed in full on pp. 23–28 of this report. The only article that appears to require comment is No. 3, establishing a Committee of Review and defining its powers. The logic of a Trust Deed seems to require provision for disposition of the funds in case of death of the party, or dissolution of the corporation, for whose benefit the deed was created. In the case of our Laboratory protection of the purposes for which we secured the funds also seemed to be demanded. The Committee of Review is established to protect the interests both of the present members of the Laboratory and also of the donors of the fund in these purposes, and in addition to ensure that the income shall be used in perpetuity for biological research. It will be noted that two thirds of the nine members of the Committee of Review are required to be Biologists nominated by Professors of Biology in the institutions named, and that it requires a two-thirds vote of the Committee twice repeated at an interval of five years to divert the funds. Members of the Laboratory are eligible to membership on the Committee of Review. The Committee is not a standing Committee; it comes into existence and disappears automatically every ten years. To divert the funds, they have to testify in writing, stating their reasons therefor, that the Laboratory is not performing valuable services in Biological Research. The Laboratory is not limited in its activities in any way; the future administration of the Laboratory is pledged to maintain research, but may also engage in any other activities authorized by the Act of Incorporation.

It should also be stated that Mr. John D. Rockefeller, Jr.'s contribution of $400,000 for endowment, and the contribution of $100,000 by the Carnegie Corporation for the same purpose, have been deposited with the same Trust Company under the same Deed of Trust in accordance with Article 13 of the Deed.

Reference was made in the Director's Report for 1921 also to the plans of the new building. We publish herewith (p. 29 ff.)

simplified drawings of the ground plans for the information of our members; in a later report it is hoped that Dr. Drew will include an adequate description of the new building and its appliances.

7. *Changes in the Membership of the Board of Trustees.*—At the meeting of the Corporation August 14, 1923, three new members of the Board of Trustees were included in the elections to the class of 1927: Professor Winterton C. Curtis of the University of Missouri, Professor J. R. Schramm of Cornell University, and the National Research Council, and Professor L. L. Woodruff of Yale University, all previously active for years in affairs of the Marine Biological Laboratory.

The deaths of the Treasurer of the Laboratory and of the Secretary of the Board of Trustees, both for many years members of the Board and conspicuous for their services to the Laboratory, were commemorated in the following memorials presented to the Corporation and to the Board:

Resolution on the death of D. Blakely Hoar, Treasurer of the Marine Biological Laboratory, presented by Professor G, H. Parker.

D. Blakely Hoar, Treasurer of the Marine Biological Laboratory, died in Boston, March 8, 1923. Mr. Hoar first came into the office of Treasurer in October, 1899, and served the Laboratory in this capacity for almost twenty-four years. He began his duties during a very trying period in which the Laboratory was reorganized, and under circumstances that must have been for him often little short of embarrassing. But he was not a man to be put down by such conditions, and from the beginning to the end of his term of service, he gave to the affairs of the Laboratory untiring interest and unswerving support. In the days of small things he exerted every effort to conserve our resources, and to make clear to many of us, who from our scientific bias may have thought otherwise, the wisdom of his course. He often expressed himself with earnest passion, yet always with a saving sense of humor and a generous patience. In him the Laboratory loses a devoted and a loyal servant, and many of its members a valued friend.

Resolution on the death of George Lefevre, Secretary of the Board of Trustees, presented by Professor M. M. Metcalf.

George Lefevre first came to Woods Hole as a Johns Hopkins University student in 1892, having a table in the Fish Commission laboratory, and he was there four summers. In 1897 he became directly connected with the Marine Biological Laboratory as an instructor in the Zoölogy course. He served in this position for two years. Since 1905 he was continuously upon the staff for direction of zoölogical research; he was fourteen years a Trustee and was Secretary of the Board for ten years. Few of the present or former members of the Woods Hole group have served the Laboratory for a longer period. Five of Dr. Lefevre's publications are based wholly or in part upon investigations conducted here. Nearly every summer some of his pupils at the University of Missouri or some of the members of their zoological staff were at our Laboratory, and for six years the University of Missouri contributed to the financial support of the Laboratory, all doubtless through Dr. Lefevre's influence.

But this bare statement of formal connections with the institution gives no adequate idea of the faithfulness of the service rendered, or of the influence of his fine personality and of his accurate work as an investigator. He was an outstanding figure in our Woods Hole group, his unfailing considerateness and courtesy, both as scientist and as friend, together with his genial sense of humor, contributing a large share to that wholesome atmosphere which has been one of the chief assets of this Laboratory, so free from personal jealousies. George Lefevre was a sound zoölogist who did much good technical scientific work; he was a keen critic, discriminating in suggestion; he was an inspiring teacher; he was an administrator of rare tact, good judgment and efficiency and our Woods Hole Laboratory, as well as his own University, had the benefit of his wise counsel. But while recognizing to the full his strength as a scientist and as a leader and the thoroughness and devotion of his service to this institution, our chief remembrance of him will be as a sensitive gentleman, a tactful counsellor and a warm-hearted friend. The keen sense of our own loss in his death prompts us to express to his colleagues at the University of Missouri and especially to the members of his family our deep sympathy.

There are included as parts of this report, 1. The Deed of Trust covering funds for endowment, 2. Plans of the new building, 3. The new form of application for admission to courses, 4. The Staff, 1923, 5. List of investigators and students, 1923, 6. A tabular view of attendance, 1919–23, 7. The subscribing and coöperating institutions, 1923, 8. The evening lectures, 9. The membership of the Corporation as of August 14, 1923.

I. THE DEED OF TRUST COVERING FUNDS FOR ENDOWMENT.

FRIENDSHIP FUND, INC., AND CENTRAL UNION TRUST COMPANY OF NEW YORK.

AGREEMENT.

DATED NEW YORK, JANUARY 22, 1924.

AGREEMENT made the 22d day of January, 1924, between FRIENDSHIP FUND, INC., a corporation organized and existing under the Membership Corporation Law of the State of New York, and having its principal office at 70 Fifth Avenue, in the City, County and State of New York, (hereinafter known as the "Grantor"), and CENTRAL UNION TRUST COMPANY OF NEW YORK, a corporation organized and existing under the Laws of the State of New York, (hereinafter sometimes called the "Trustee"), having its principal office at 80 Broadway, in the City, County and State of New York:

WITNESSETH:

That in consideration of the premises and of the agreements hereinafter contained, the Grantor does hereby assign, transfer and set over unto the said Central Union Trust Company of New York, as Trustee, and to its successor and successors in trust, securities of the par value of Four hundred and five thousand Dollars ($405,000), and of the approximate market value as of December 31, 1923, of Three hundred ninety four thousand and twenty-one Dollars ($394,021), which said securities are listed upon a schedule hereto annexed and marked list of securities, IN TRUST NEVERTHELESS, upon the uses and trusts following, that is to say:

1. To hold, manage, invest and reinvest the same, to collect the income, interests, issues and profits therefrom and after paying all proper and necessary expenses including compensation of the Trustee to pay over the net income as collected in monthly instalments to the Marine Biological Laboratory, (sometimes hereinafter called the "Beneficiary"), a corporation organized and existing under the Laws of the State of Massachusetts, and having at the present time its

laboratory at Woods Hole, in the Town of Falmouth, State of Massachusetts. All such payments shall be made to the Treasurer of the Beneficiary or such other officer or person on its behalf, as the said Beneficiary may from time to time designate.

2. The investment and reinvestment of the fund shall be made by the Trustee in consultation with the Treasurer of the Beneficiary.

The Trustee shall be at liberty to retain any of the securities received by it hereunder, and it may in its discretion sell all or any part of the same, but in making reinvestments of such fund it shall be limited to securities permitted to Trustees by the Laws of any of the States of the United States.

3. A Committee of Review is hereby constituted. Such Committee to consist of nine (9) members in all and to include an official representative of each of the following: The National Academy of Sciences, the National Research Council, and the American Association for the Advancement of Science, and six (6) professors in the field of biology named respectively by a majority vote of the professors of the departments in the field of biology of the following universities, to wit: Harvard University, Columbia University, University of Chicago, Princeton University, Yale University, and University of Pennsylvania.

Membership on the Board of Trustees of the Marine Biological Laboratory or other connection with the Beneficiary shall not disqualify a person from acting upon said Committee of Review.

In the event that any of the nine institutions mentioned shall be unwilling or unable to appoint a representative, such vacancy shall be filled by an appointment made in the same manner as above provided by the departments in the field of biology of the following universities, in order named: University of Michigan, Johns Hopkins University and Leland Stanford University, and in the event of any of these universities being unwilling or unable to fill such vacancy, or of there being any further vacancies in said Committee, then such vacancy or vacancies may be filled by appointment of professors in the field of biology named by the majority vote of the members of the Committee of Review already appointed.

It shall be the duty of the Trustee to call once every ten years (10) upon this Committee of Review, to make a study of the work at the Marine Biological Laboratory, and if two-thirds of the entire Committee shall agree that the Marine Biological Laboratory is not performing valuable services in biological research and shall file a written memorandum with the Trustee stating such conclusion and their reasons therefor, a copy of which memorandum shall be sent to the

Beneficiary forthwith, and if after a period of five years two-thirds of the entire Committee of Review as then constituted shall again report in writing to the Trustee that the Marine Biological Laboratory is not performing valuable services in biological research, then the fund both principal and interest may be used for such other purposes within the general field of biological research as shall be indicated by this Committee of Review, on an affirmative vote of not less than two-thirds of the entire membership of said Committee.

4. The said Committee of Review may by two-thirds vote of its entire membership, remove the Trustee, and upon the removal, resignation or cessation of the Trustee to function, may appoint a successor trustee, and in default of such appointment the duty of appointing a successor trustee shall devolve upon the Supreme Court of the State of New York. The Beneficiary may call the Committee together for such action.

5. The Trustee shall send semi-annually to the Treasurer of the Beneficiary, a full statement of the investments then held by the Trustee, and of the receipts and expenditures in both income and principal accounts since the last semi-annual statement, and at the request of the Treasurer, the Trustee shall cause an audit to be made of the books and accounts of the Trustee with respect to this trust, by certified accountants of recognized standing, and a reasonable compensation for such audit if requested by the Treasurer, shall be paid by the Trustee and charged against the income as an expense of the administration of the trust.

6. The Trustee is authorized and empowered as the Trustee of an express trust to institute, conduct, defend, intervene in and compromise or terminate any and all suits, actions or proceedings at law or in equity or otherwise whenever the Trustee shall consider it necessary or proper so to do for the collection of the principal or interest on any of the securities held by it hereunder, or for the securing or protecting or defense of any rights or interests relating to or with respect to the property held in trust hereunder, or of the rights or interests of itself as Trustee, or of the Beneficiary hereunder with respect to the trust property. The Trustee shall have full discretionary power to allocate as between principal and income any expense with respect to the foregoing, and shall have full discretionary power to sell such portion of the trust as may be necessary to pay or recoup itself for the payments of such amount of such expense as shall have been allocated to the principal or the trust estate.

7. The Trustee is authorized and empowered as the Trustee of an express trust, to exercise all rights and powers pertaining to any

securities held by it; and as the holder of such securities the Trustee may in its discretion join in any reorganization, merger, consolidation or dissolution of any corporation whose securities it holds, whenever such action may be deemed by the Trustee in its discretion necessary or proper in the interests of the trusts; and the Trustee may accept in exchange for such securities any new securities issued in exchange therefore upon any such reorganization, merger, consolidation or dissolution.

The provisions of this article are not intended as a limitation upon the powers accruing to the Trustee hereunder as a Trustee of an express trust.

8. The Trustee shall not be required to retain or set aside any portion of the income derived from securities for the purpose of creating a sinking fund to amortize the premium upon any securities purchased or taken over above par and conversely, if securities shall be purchased or acquired at a discount, and there shall subsequently be collected or realized thereon on sale or collection an amount in excess of the original investment therein, the amount of such excess shall be treated for all purposes as principal and not income.

9. The Trustee shall be entitled to reasonable compensation for its services rendered hereunder, and as long as the Central Union Trust Company of New York shall be Trustee it is agreed that the amount of such compensation in full for all its services herein shall be the sum of Three hundred and fifty Dollars ($350), per annum. Payment on account of such amount to be deducted and withheld from time to time in the discretion of the Trustee as income is collected or received.

10. The Grantor reserves unto itself full power to enforce the provisions of this Deed or Trust, and, for that purpose, to institute, conduct, defend, intervene in, compromise or terminate, either by itself or jointly with the Beneficiary, any and all suits, actions or proceedings at law or in equity or otherwise against the Trustee or its successors under this Deed of Trust, provided that the Grantor, when acting under the provisions of this article shall be deemed to be acting on behalf of the Beneficiary; provided further, that nothing in this article shall be deemed to deprive the Beneficiary named herein, or the person or persons to whom the net income and revenue arising under this Trust are payable, of the right to enforce in behalf of the Beneficiary the provisions of this Deed of Trust, except in cases in which the Grantor shall already have taken action to enforce such provisions on behalf of the Beneficiary.

11. Except in so far as is otherwise provided by the provisions of this Deed of Trust, the Trustee named shall be deemed to possess and

enjoy the powers and rights recognized under the law of the State of New York as belonging to the Trustee of an express trust.

12. This deed may be amended by agreement in writing between the Friendship Fund, the Trustee for the time being and the Beneficiary, or if the Friendship Fund has ceased to exist by the agreement of the Trustee and the Beneficiary, with the approval of two thirds of the entire membership of the Committee of Review.

13. The Trustee is authorized to receive and hold under this Deed additional securities, cash and property, pursuant to the terms and conditions hereof. Such additional gifts may be received from any person, corporation or association. The compensation of the Trustee on such additions shall be proportionate to the compensation herein provided.

IN WITNESS WHEREOF, the FRIENDSHIP FUND, INC., has caused these presents to be executed by its duly authorized officers, and the CENTRAL UNION TRUST COMPANY OF NEW YORK, in token of its acceptance of the trusts herein contained, has caused these presents to be executed by its duly authorized afficers, as of the 22d day of January, 1924.

FRIENDSHIP FUND, INC.,
(Signed) LAWRASON RIGGS, JR., Signed by CHARLES R. CRANE,
Secretary. *President,*
LAWRASON RIGGS, JR.,
Treasurer

CENTRAL UNION TRUST COMPANY OF NEW YORK,
Signed by B. A. MORTON,
Vice-President.

Attest:
H. L. WILLIAMS,
Asst. Secretary.
(SEAL)

WHEREAS, FRIENDSHIP FUND, INC., did on the 22d day of January, 1924, make a Deed of Trust to the CENTRAL UNION TRUST COMPANY OF NEW YORK, as Trustee, of certain securities for the benefit of Marine Biological Laboratory, as is more fully set forth in said Deed; and

WHEREAS, paragraph 13 of said Deed provides as follows:

> "13. The Trustee is hereby authorized to receive and hold under this Deed additional securities, cash and property pursuant to the terms and conditions hereof. Such additional gifts may be received from any person, corporation or association. The

compensation of the Trustee on such additions shall be proportionate to the compensation herein provided."

KNOW ALL MEN BY THESE PRESENTS:

That in consideration of the premises and in consideration of the payment to it as Trustee, on January 25th, 1924, of $400,000 from Mr. John D. Rockefeller, Jr., by check to the order of Marine Biological Laboratory, endorsed to Central Union Trust Company of New York, as Trustee, under said Deed aforementioned, and in consideration of the payment to it as Trustee, on January 26th, 1924 of $100,000 from Carnegie Corporation of New York, by check to the order of Marine Biological Laboratory, endorsed to the Central Union Trust Company of New York, as Trustee, receipt whereof is hereby acknowledged, the CENTRAL UNION TRUST COMPANY OF NEW YORK agrees with Friendship Fund, Inc., and with the other donors above mentioned, to hold said additional gifts under all of the terms and conditions of said Deed of Trust between Friendship Fund, Inc. and Central Union Trust Company of New York, dated January 22, 1924.

IN WITNESS WHEREOF, Central Union Trust Company of New York, has caused these presents to be executed by its duly authorized officers and its corporate seal to be hereunto affixed, as of the 26th day of January, 1924.

CENTRAL UNION TRUST COMPANY OF NEW YORK,
By B. A. MORTON.
Vice-President. (SEAL)

STATE OF NEW YORK }
COUNTY OF NEW YORK } *ss.:*

On this 4th day of February, in the year One thousand nine hundred and twenty-four, before me, the undersigned, personally came B. A. MORTON, to me known, who, being by me duly sworn, did depose and say that he resides in New York, N. Y.; that he is a Vice President of CENTRAL UNION TRUST COMPANY OF NEW YORK, the corporation described in and which executed the foregoing instrument; and that he signed his name thereto by order of the Board of Trustees of said corporation.

HAROLD A. CUNNINGHAM. (SEAL)
Notary Public.

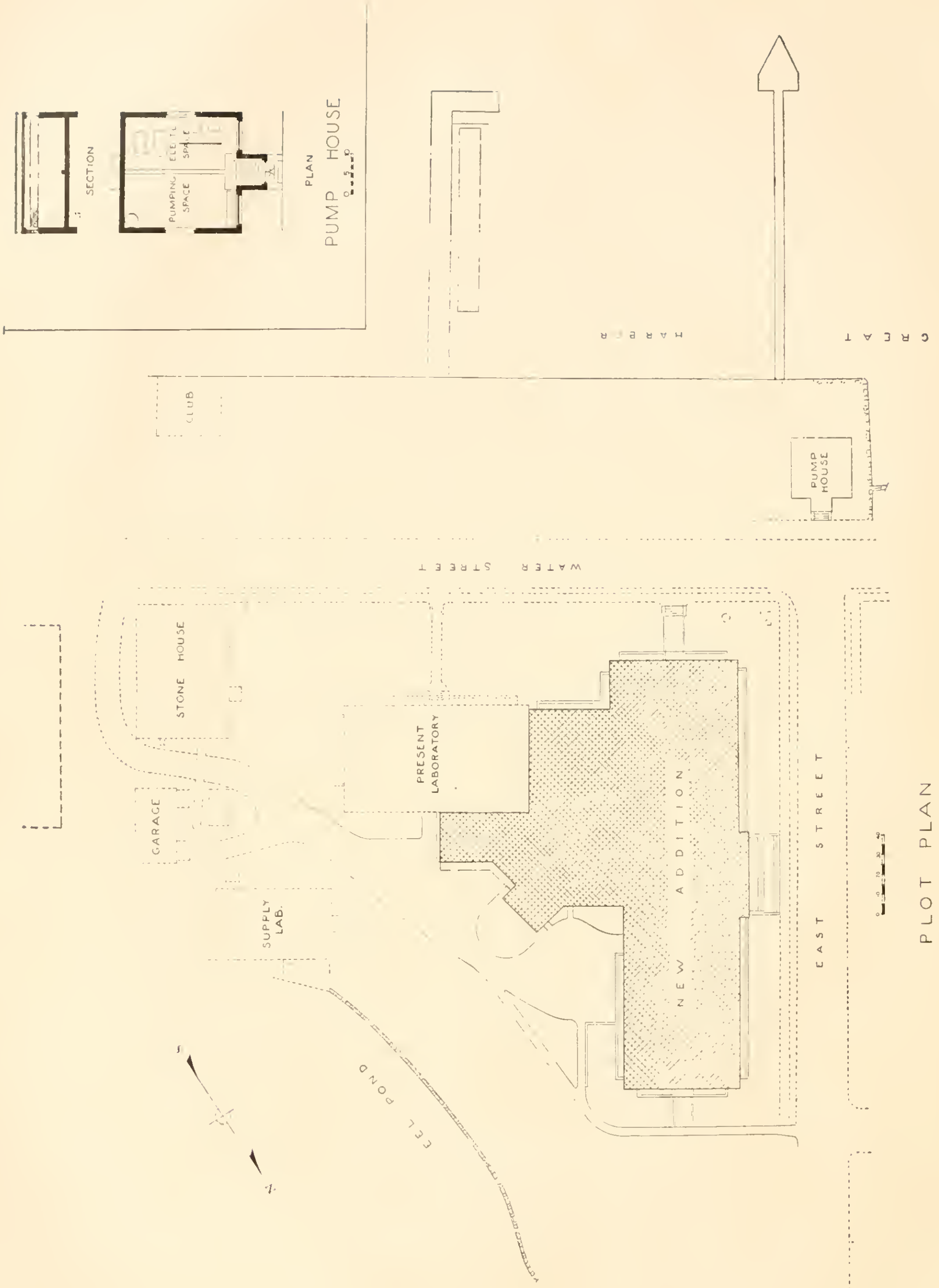
2. PLANS OF THE NEW BUILDING.

The new building faces about South-west; see plan 1.

The uses of the various rooms are indicated by specific designations on the plans. The laboratories not otherwise designated are designed for the following purposes: (1) Primarily for biological research—120, 123, 140, 141, 206, 208, 209, 217, 218, 229, 235, 236, 238, 239, 240, 241, 242, 315, 318, 320, 322, 325, 326, 329, 330, 341; (2) for biochemical research—121, 122, 124, 142, 207, 219, 221, 316, 317, 321, 327, 328, 342, 343, 344, 345; (3) for biophysical research—126, 311, 312, 313, 315, 339.

The biological laboratories are equipped with running salt and fresh water, direct and alternating electric current, gas and the usual laboratory furniture. The biochemical laboratories have in addition chemical hood and desk with the usual appliances. The two general biophysical laboratories, 126 and 339, have the fittings of the biological laboratories, apparatus case and open space for setting up apparatus; the X-ray rooms are protected by lead-sheathed walls.

The roof-plan shows the intention of storing the sea-water in shallow cement tanks not visible from the ground which will replace the tall wooden tanks on the present building.


SECTION
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PLAN
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CLUB
HARBOR
GREAT
PUMP HOUSE
WATER STREET
STONE HOUSE
PRESENT LABORATORY
GARAGE
SUPPLY LAB.
NEW ADDITION
EAST STREET
EEL POND
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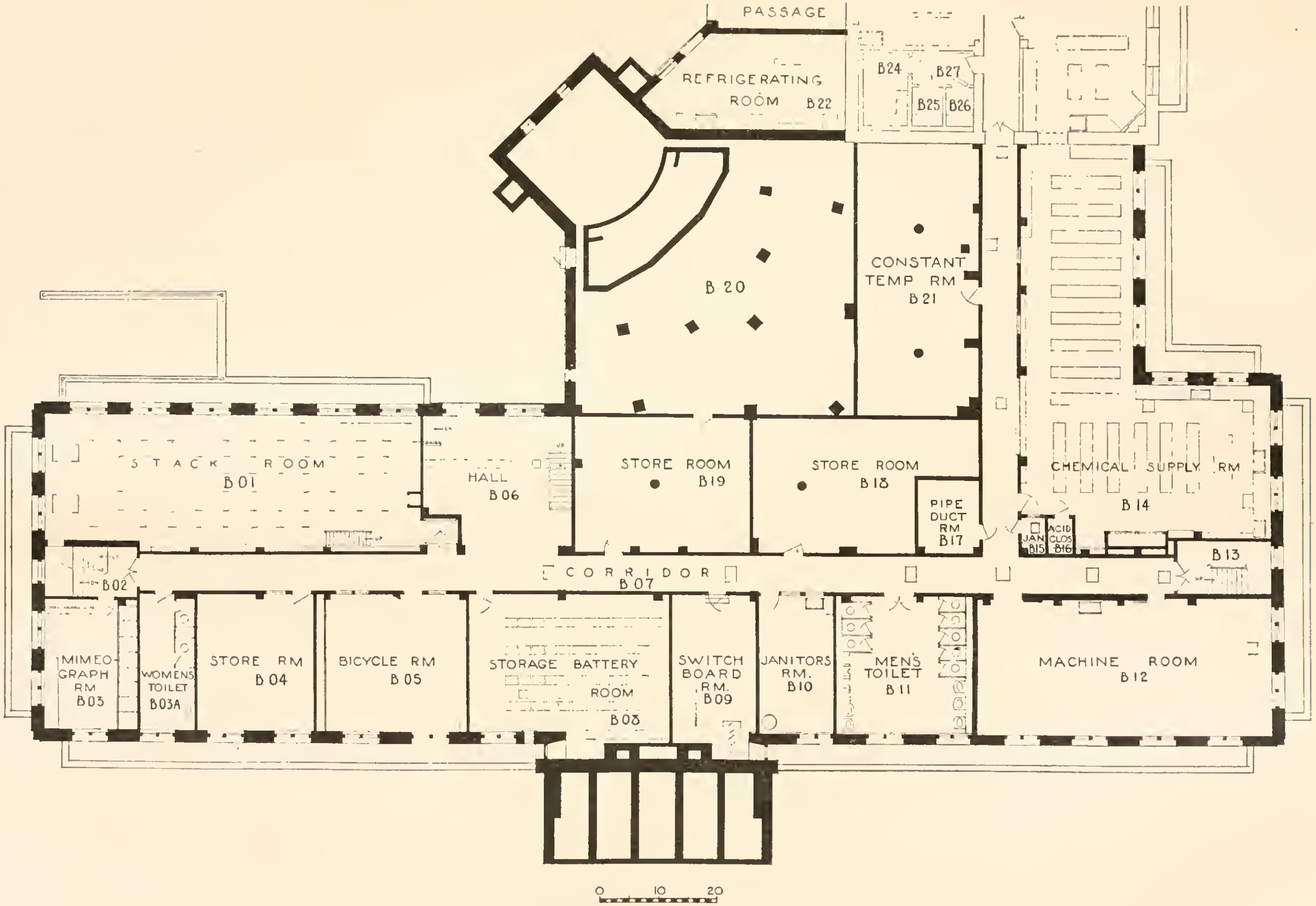
PLOT PLAN


PASSAGE
REFRIGERATING ROOM B22
B24
B27
B25
B26
B 20
CONSTANT TEMP RM B21
CHEMICAL SUPPLY RM B14
JAN B15
ACID CLOS B16
B13
STACK ROOM B01
HALL B06
STORE ROOM B19
STORE ROOM B18
PIPE DUCT RM B17
B02
CORRIDOR B07
MIMEO-GRAPH RM B03
WOMENS TOILET B03A
STORE RM B04
BICYCLE RM B05
STORAGE BATTERY ROOM B08
SWITCH BOARD RM. B09
JANITORS RM. B10
MENS TOILET B11
MACHINE ROOM B12
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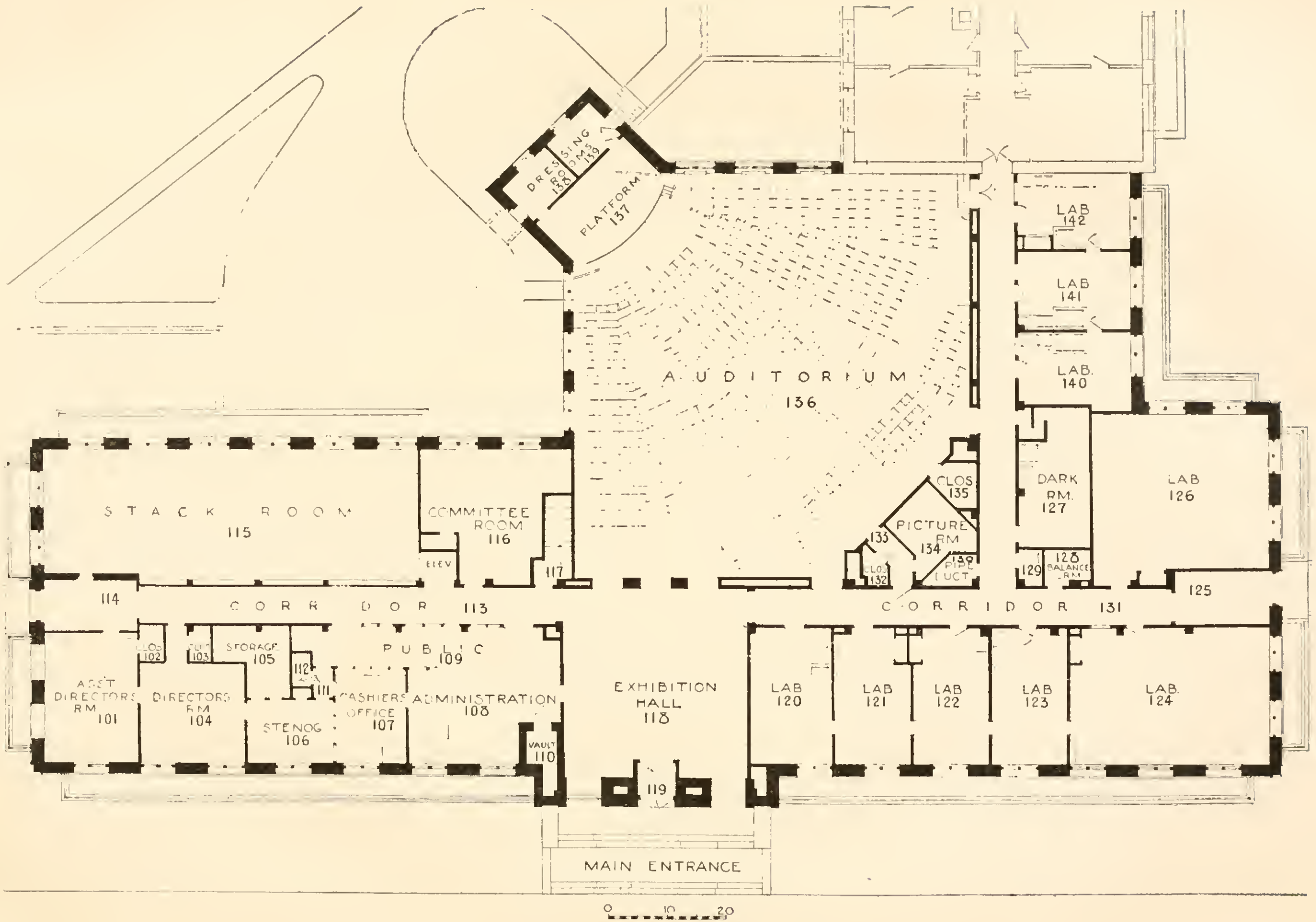
20

BASEMENT PLAN


DRESSING ROOMS 138 139
PLATFORM 137
AUDITORIUM 136
LAB 142
LAB 141
LAB. 140
STACK ROOM 115
COMMITTEE ROOM 116
ELEV
117
CLOS 135
PICTURE RM 134
133
CLOS 132
139 PIPE DUCT
DARK RM. 127
129
128 BALANCE RM
LAB 126
125
114
CORRIDOR 113
CORRIDOR 131
CLOS 102
CLOS 103
STORAGE 105
PUBLIC 109
112
111
ASST DIRECTORS RM 101
DIRECTORS RM 104
STENOG 106
CASHIERS OFFICE 107
ADMINISTRATION 108
VAULT 110
EXHIBITION HALL 118
119
LAB 120
LAB 121
LAB 122
LAB 123
LAB. 124
MAIN ENTRANCE
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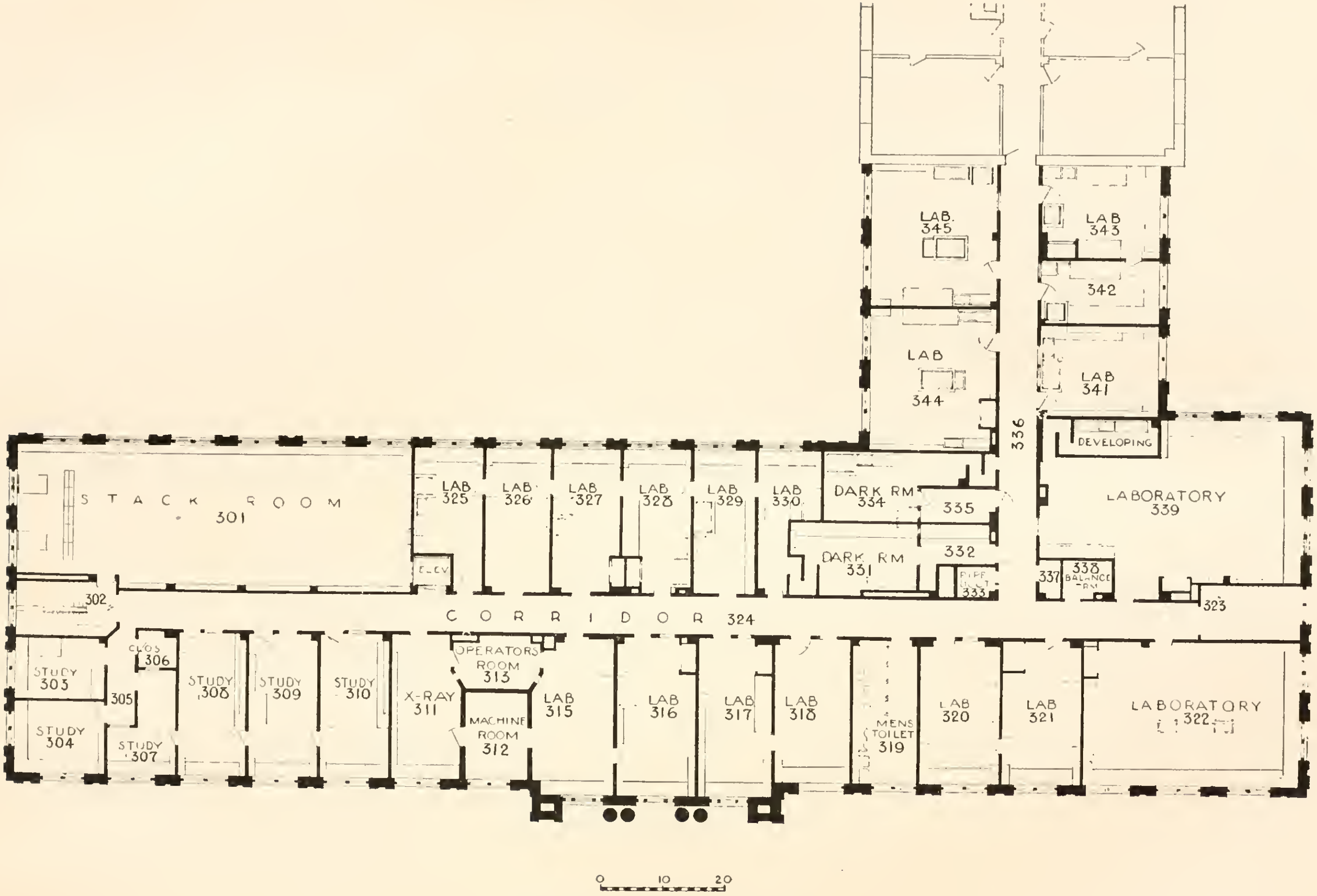
10
20

FIRST FLOOR PLAN


STACK ROOM 301
302
LAB 325
LAB 326
LAB 327
LAB 328
LAB 329
LAB 330
DARK RM 334
335
DARK RM 331
332
PIPE DUCT 333
ELEV
LAB. 345
LAB 344
LAB 343
342
LAB 341
336
DEVELOPING
LABORATORY 339
337
338 BALANCE RM
323
CORRIDOR 324
STUDY 303
STUDY 304
305
CLOS 306
STUDY 307
STUDY 308
STUDY 309
STUDY 310
X-RAY 311
OPERATORS ROOM 313
MACHINE ROOM 312
LAB 315
LAB 316
LAB 317
LAB 318
MENS TOILET 319
LAB 320
LAB 321
LABORATORY 322
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10
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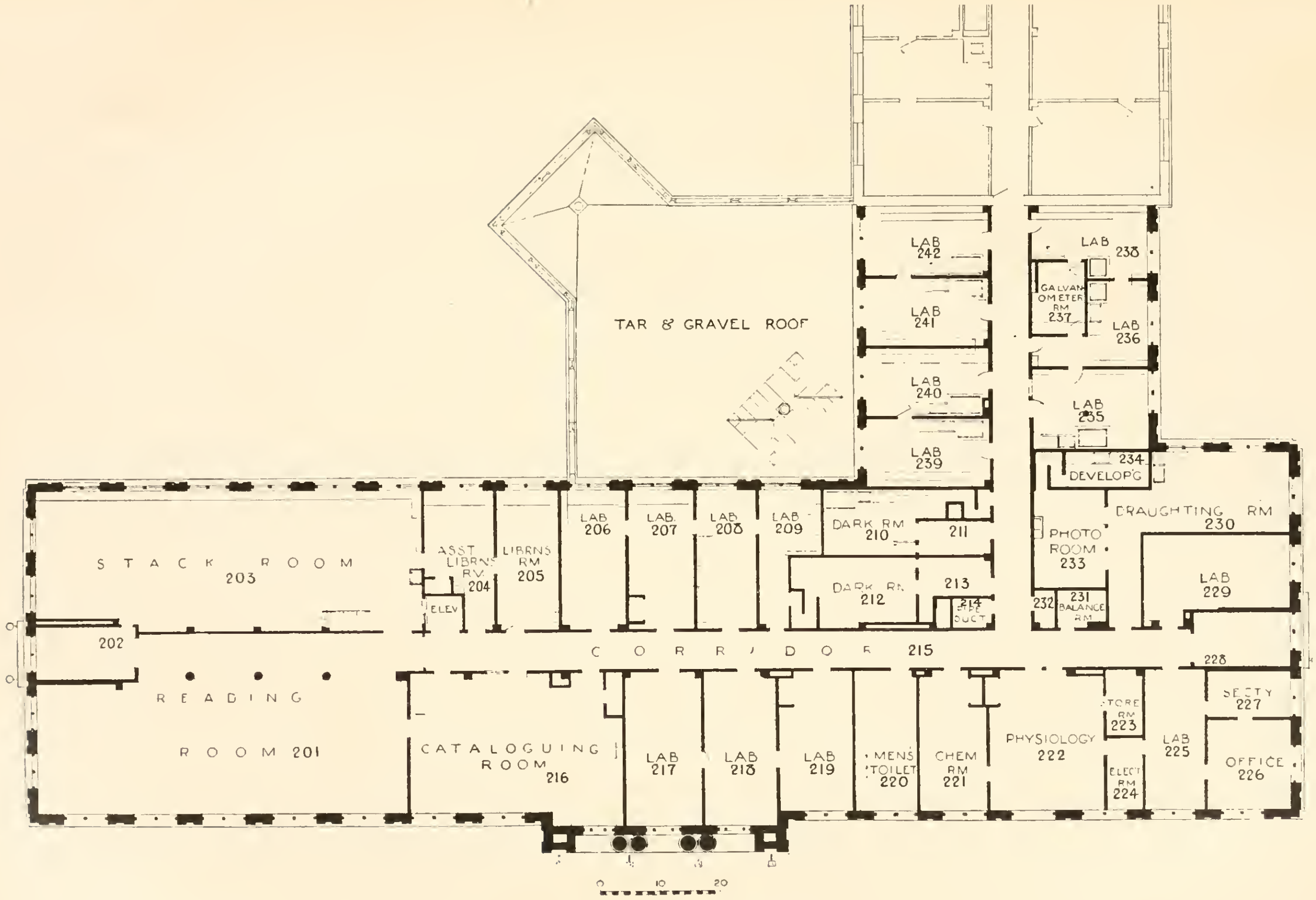
THIRD FLOOR PLAN


TAR & GRAVEL ROOF
LAB 242
LAB 241
LAB 240
LAB 239
LAB 238
GALVANOMETER RM 237
LAB 236
LAB 235
234 DEVELOP'G
STACK ROOM 203
202
ASST LIBRN'S RM 204
ELEV
LIBRNS RM 205
LAB 206
LAB 207
LAB 208
LAB 209
DARK RM 210
211
DARK RM 212
213
214 PIPE DUCT
DRAUGHTING RM 230
PHOTO ROOM 233
232
231 BALANCE RM
LAB 229
CORRIDOR 215
228
READING ROOM 201
CATALOGUING ROOM 216
LAB 217
LAB 218
LAB 219
MENS TOILET 220
CHEM RM 221
PHYSIOLOGY 222
STORE RM 223
ELECT RM 224
LAB 225
SEC'TY 227
OFFICE 226
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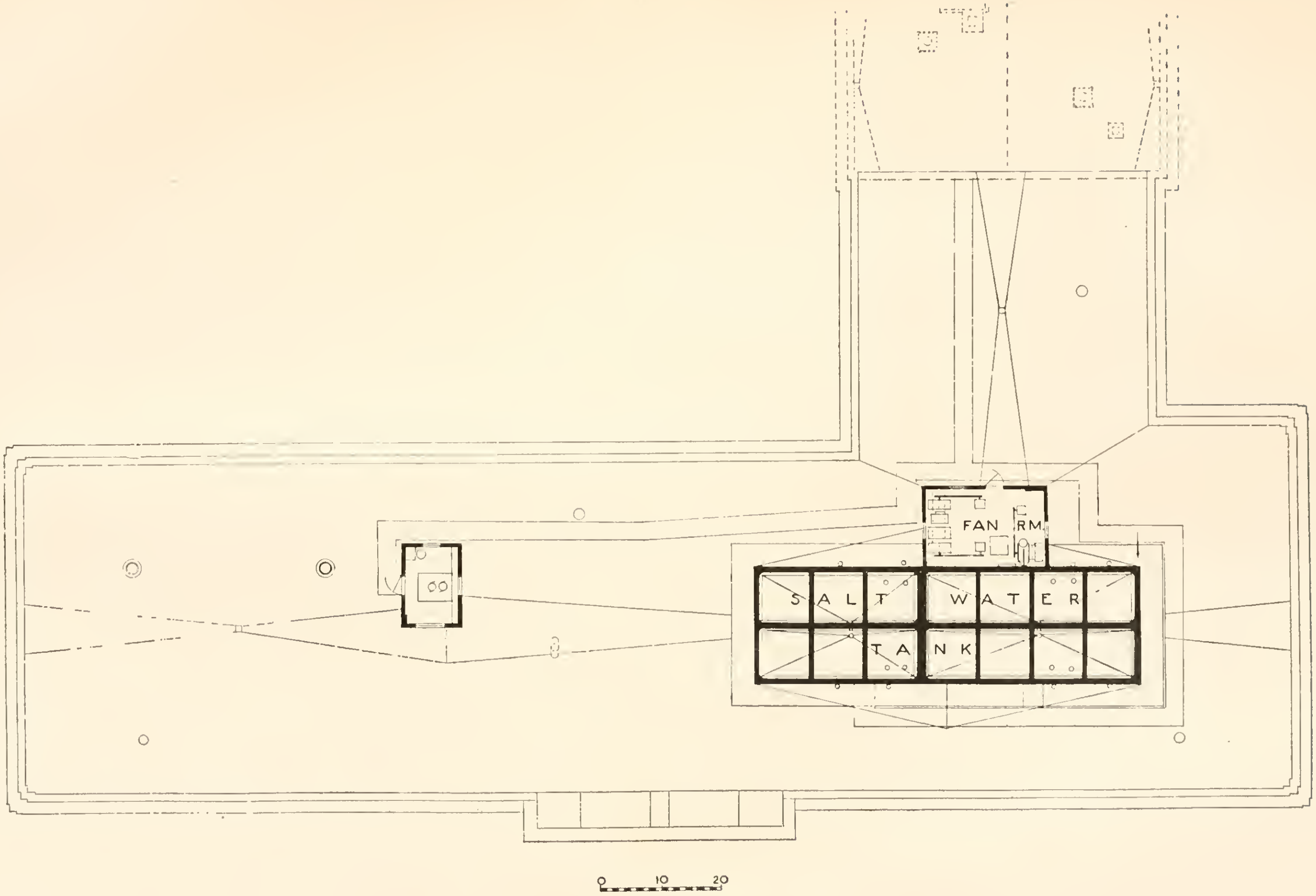
SECOND FLOOR PLAN


FAN RM
SALT WATER TANK
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20

ROOF PLAN

3. THE NEW FORM OF APPLICATION FOR ADMISSION TO COURSES.

MARINE BIOLOGICAL LABORATORY

APPLICATION FOR ADMISSION TO COURSE OF INSTRUCTION

The Laboratory assigns no grades in courses and sets no examinations. The Laboratory will therefore not certify to attainments in any course, but individual instructors are at liberty to do so on their own responsibility.

To the applicant:—Please supply the information called for on pages 1 and 2 and mail separately to the Laboratory; detach pages 3 and 4 and give to your chief instructor in biology with the request to fill in and mail directly to the Laboratory. Applicants occupying independent positions may omit pages 3 and 4, or secure endorsement according to their own judgment.

1. Name in full..

2. Course desired...

N. B. Note statement of prerequisites in the Annual Announcement.

3. Date of birth..................place of birth.................

4. Present address...

5. Permanent address..

6. Sex of applicant..

7. Nationality..................Citizenship..................

8. Institution represented.......................................

9. Status: year of undergraduate or graduate work, or official position..

....

10. College degree, with year and name of institution............

....

11. Graduate degree, with year and name of institution..........

....

12. Give list of undergraduate courses taken in chemistry, physics, and biology (including botany, zoölogy, anatomy, physiology, etc.)..

....

....

....

....

12*a*. Where taken?..

13. Similar list of graduate courses............................

....

....

....

14. List of publications and research in progress.................

....

....

....

15. State clearly reasons for wishing to take the course, and its place in your professional intention...........................

....

....

Signature......................

To the instructor or endorser of this application:—Please note the statement at the head of applicant's form. The purpose of the Marine Biological Laboratory in offering courses is to aid in the production and training of investigators; it is the intention, therefore, to give first consideration to persons who appear likely by their intellectual gifts and intentions to contribute to the advancement of science whether in biology in the stricter sense, or in medicine, or in any field in which professional biological qualification is an important factor. The status of the applicant, whether as undergraduate or graduate, may thus be of secondary importance, but other things being equal, preference will naturally be given to the better prepared student.

1. Name and address of applicant recommended................

....

2. If more than one candidate is being recommended for admission to courses from your institution, state definitely the order in which you recommended acceptance........................

....

....

3. State whether the fee of the candidate, if accepted, is to be paid by the institution.................................

....

4. State clearly your reasons for believing that the candidate possesses promise as an investigator...........................

....

....

4. THE STAFF, 1923.

FRANK R. LILLIE, *Director*, Professor of Embryology, and Chairman of the Department of Zoölogy, The University of Chicago.

GILMAN A. DREW, *Assistant Director*, Marine Biological Laboratory.

ZOÖLOGY

I. INVESTIGATION

GARY N. CALKINS, Professor of Protozoölogy, Columbia University.

E. G. CONKLIN, Professor of Zoölogy, Princeton University.

CASWELL GRAVE, Professor of Zoölogy, Washington University.

H. S. JENNINGS, Professor of Zoölogy, Johns Hopkins University.

FRANK R. LILLIE, Professor of Embryology, the University of Chicago.

C. E. MCCLUNG, Professor of Zoölogy, University of Pennsylvania.

S. O. MAST, Professor of Zoölogy, John Hopkins University.

T. H. MORGAN, Professor of Experimental Zoölogy, Columbia University.

G. H. PARKER, Professor of Zoölogy, Harvard University.

E. B. WILSON, Professor of Zoölogy, Columbia University.

II. INSTRUCTION

ROBERT H. BOWEN, Assistant Professor of Zoölogy, Columbia University.

EDWARD F. ADOLPH, Instructor in General Physiology, University of Pittsburgh.

J. A. DAWSON, Assistant Professor of Biology, Dalhousie University.

S. W. GEISER, Assistant Professor of Zoölogy, Washington University.

ANN H. MORGAN, Professor of Zoölogy, Mount Holyoke College.

J. PAUL VISSCHER, U. S. Bureau of Fisheries. (Absent on leave.)

B. H. WILLIER, Instructor in Zoölogy, The University of Chicago.

BENJAMIN P. YOUNG, Assistant Professor of Zoölogy, Cornell University.

DONNELL B. YOUNG, Assistant Professor of Biology, Carleton College.

PROTOZOÖLOGY

I. INVESTIGATION

(*See Zoölogy*)

II. INSTRUCTION

GARY N. CALKINS, Professor of Protozoölogy, Columbia University.

LOUISE H. GREGORY, Assistant Professor of Zoölogy, Columbia University. (Absent 1923.)

FLORENCE DE L. LOWTHER, Instructor in Zoölogy, Barnard College.

EMBRYOLOGY

I. INVESTIGATION

(*See Zoölogy*)

II. INSTRUCTION

HUBERT B. GOODRICH, Associate Professor of Zoölogy, Wesleyan University.

BENJAMIN H. GRAVE, Professor of Biology, Wabash College.

HAROLD H. PLOUGH, Associate Professor of Biology, Amherst College.

CHARLES G. ROGERS, Professor of Comparative Physiology, Oberlin College.

ELIZABETH A. SMITH, Assistant Professor of Zoölogy, University of Wisconsin.

PHYSIOLOGY

I. INVESTIGATION

HAROLD C. BRADLEY, Professor of Physiological Chemistry, University of Wisconsin.

Walter E. Garrey, Professor of Physiology, Tulane University.

Ralph S. Lillie, Biologist, Nela Research Laboratories, Department of Pure Science, Nela Park, Cleveland, Ohio.

Albert P. Mathews, Professor of Biochemistry, The University of Cincinnati.

II. INSTRUCTION

Merkel H. Jacobs, Assistant Professor of Physiology, University of Pennsylvania.

William R. Amberson, Assistant Professor of Physiology, College of Medicine, University of Tennessee.

Frank P. Knowlton, Professor of Physiology, Syracuse University.

Reynold A. Spaeth, Associate in Physiology, School of Hygiene and Public Health, Johns Hopkins University.

BOTANY

I. INVESTIGATION

S. C. Brooks, Department of Public Health, Washington, D. C.

Edward M. East, Professor of Experimental Plant Morphology, Harvard University.

Robert A. Harper, Professor of Botany, Columbia University.

E. Newton Harvey, Assistant Professor of Physiology, Princeton University.

Winthrop J. V. Osterhout, Professor of Botany, Harvard University.

Jacob R. Schramm, Professor of Botany, College of Agriculture, Cornell University.

II. INSTRUCTION

Ivey F. Lewis, Professor of Biology, University of Virginia.

William Randolph Taylor, Assistant Professor of Botany, University of Pennsylvania.

William H. Weston, Jr., Assistant Professor of Cryptogamic Botany, Harvard University.

LIBRARY

Robert P. Bigelow, Librarian and Professor of Zoölogy and Parasitology, Massachusetts Institute of Technology. Librarian.

Priscilla B. Montgomery (Mrs. Thomas H. Montgomery, Jr.), Assistant Librarian.

CHEMICAL SUPPLIES

Oliver S. Strong, Associate Professor of Neurology, Columbia University, New York City, Chemist.

SUPPLY DEPARTMENT

George M. Gray, Curator.
Thomas M. Douthart, Assistant Curator.
John J. Veeder, Captain.
E. M. Lewis, Engineer.
A. W. Leathers, Head of Shipping Department.
Milton B. Gray, Collector.
A. M. Hilton, Collector.
J. McInnis, Collector.

F. M. MacNaught, Business Manager.
Herbert A. Hilton, Superintendent of Buildings and Grounds.

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5. INVESTIGATORS AND STUDENTS, 1923.

Adams, A. Elizabeth, Associate Professor of Zoölogy, Mount Holyoke College.
Adolph, Edward F., Instructor in Zoölogy, University of Pittsburgh.
Agersborg, H. P. K., Instructor in Biology, Williams College.
Bowen, Robert H., Assistant Professor of Zoölogy, Columbia University.
Bridges, Calvin B., Research Assistant, Carnegie Institution of Washington.
Calkins, Gary N., Professor of Zoölogy, Columbia University.
Chambers, Robert, Professor of Microscopic Anatomy, Cornell University Medical College.
Child, C. M., Professor of Zoölogy, University of Chicago.
Clapp, Cornelia M., Professor Emeritus of Zoölogy, Mount Holyoke College.
Clark, Eliot R., Professor of Anatomy, University of Georgia.
Clark, Eleanor L., 1104 Milledge Road, Augusta, Ga.
Cole, Arch E., Assistant Professor of Zoölogy, Northwestern University.
Cole, William H., Professor of Biology, Lake Forest College.
Conklin, Edwin G., Professor of Biology, Princeton University.
Copeland, Manton, Professor of Biology, Bowdoin College.
Cowdry, Edmund V., Associate Member, Rockefeller Institute.
Cowdry, Nathaniel H., 1142 Madison Ave., New York City.
Curtis, Winterton C., Professor of Zoölogy, University of Missouri.
Danchakoff, Vera, Assistant Professor of Anatomy, College of Physicians and Surgeons.
Dawson, James A., Instructor in Zoölogy, Harvard University.
Degener, Lyda M., 5002 Penn St., Frankford, Pa.
Detwiler, Samuel R., Assistant Professor, Harvard University.
Dolley, William L., Jr., Professor of Biology, Randolph-Macon College.
Donaldson, Henry H., Professor of Neurology, Wistar Institute.
Drew, Gilman A., Assistant Director, Marine Biological Laboratory, Woods Hole, Mass.
Fischelis, Philipp, Professor of Histology and Embryology, Temple University.
Geiser, Samuel W., Assistant Professor of Zoölogy, Washington University.
Glaser, Otto C., Professor of Biology, Amherst College.
Goodrich, Hubert B., Professor of Biology, Wesleyan University.
Grave, Benjamin H., Professor of Zoölogy, Wabash College.
Grave, Caswell, Professor of Zoölogy, Washington University.

Guthrie, Mary J., Assistant Professor, University of Missouri.

Hegner, Robert W., Professor of Protozoölogy, School of Hygiene and Public Health, Johns Hopkins University.

Heilbrunn, Lewis V., Assistant Professor of Zoölogy, University of Michigan.

Hess, Walter N., Professor of Biology, De Pauw University.

Hibbard, Hope, Associate Professor of Zoölogy, Elmira College.

Hinrichs, Marie A., Research Assistant, Nela Research Laboratories, Cleveland, Ohio.

Hitchcock, David I., Associate, Rockefeller Institute for Medical Research.

Hogue, Mary J., Professor of Bacteriology, North Carolina College for Women.

Holt, L. Emmett, Jr., Rockefeller Institute for Medical Research.

Huettner, Alfred F., Instructor, Columbia University.

Jennings, Herbert S., Professor of Zoölogy, Johns Hopkins University.

Just, Ernest E., Professor of Zoölogy, Howard University.

Kindred, James E., University of Virginia.

Knower, Henry McE., Professor of Anatomy, University of Cincinnati.

Komai, Taku, Assistant Professor of Zoölogy, Kyoto Imperial University.

Lancefield, Donald E., Assistant Professor of Zoölogy, Columbia University.

Lewis, Warren H., Research Associate, Carnegie Institution of Washington.

Lewis, Margaret R., Collaborator, Carnegie Institution of Washington.

Lillie, Frank R., Chairman, Department of Zoölogy, University of Chicago.

Lynch, Ruth S., Assistant in Graduate Zoölogy, Johns Hopkins University.

McClung, Clarence E., Director of Zoölogical Laboratory, University of Pennsylvania.

Malone, Edward F., Professor of Histology, University of Cincinnati.

Martin, Earl A., Assistant Professor, College of the City of New York.

Mast, Samuel O., Professor of Zoölogy, Johns Hopkins University.

Mavor, James W., Associate Professor of Biology, Union College.

Metcalf, Maynard M., 128 Forest St., Oberlin, Ohio.

Metz, Charles W., Member of Staff, Carnegie Institution, Cold Spring Harbor.

Miner, Roy W., Curator of Lower Invertebrates, American Museum of Natural History.

Minnich, Dwight E., Assistant Professor of Animal Biology, University of Minnesota.

Morgan, Thomas H., Professor of Experimental Zoölogy, Columbia University.

Morgan, Lilian V., 409 W. 117th St., New York City.

Morgan, Ann H., Professor of Zoölogy, Mount Holyoke College.

Parker, George H., Professor of Zoölogy, Harvard University.

Parmenter, Charles L., Instructor, University of Pennsylvania.

Peebles, Florence, Pineville, Pa.

Phillips, Everett F., U. S. Dept. of Agriculture, Washington, D. C.

Plough, Harold H., Associate Professor of Biology, Amherst College.

Rand, Herbert W., Associate Professor of Zoölogy, Harvard University.

Rogers, Charles G., Professor of Comparative Physiology, Oberlin College.

Sampson, Myra M., Assistant Professor of Zoölogy, Smith College.

Schrader, Franz, Bryn Mawr College.

Smith, Elizabeth A., Assistant Professor of Zoölogy, University of Wisconsin.

Stockard, Charles R., Professor of Anatomy, Cornell University Medical College.

Strong, Oliver S., Associate Professor of Neurology, Columbia University.

Sturtevant, Alfred H., Member of Staff, Carnegie Institution of Washington.

Swett, Francis H., Instructor, Johns Hopkins University Medical School.

Weinstein, Alexander, Research Fellow, Johns Hopkins University.
Wenrich, David H., Assistant Professor of Zoölogy, University of Pennsylvania.
Whedon, Arthur D., Head of Zoölogy Department, North Dakota Agricultural College.
White, Gertrude M., Instructor, Carnegie Institute of Technology.
Wislocki, George B., Associate Professor of Anatomy, Johns Hopkins University.
Whiting, Phineas W., University of Iowa.
Wieman, Harry L., Professor of Zoölogy, University of Cincinnati.
Willier, B. H., Instructor in Zoölogy, University of Chicago.
Wilson, Edmund B., Professor of Zoölogy, Columbia University.
Woodruff, Lorande L., Professor of Protozoölogy, Yale University.
Woodward, Alvalyn E., North Carolina College for Women.
Young, Benjamin P., Assistant Professor of Zoölogy, Cornell University.
Young, Donnell B., Assistant Professor of Biology, Carleton College.

BEGINNING INVESTIGATORS—Zoölogy.

Austin, Mary L., Columbia University.
Barrett, Elizabeth, Student, University of Chicago.
Bean, Raymond J., Instructor in Biology, Western Reserve University.
Beerman, Herman, University of Pennsylvania.
Bollman, Loretta M., Notre Dame College.
Botsford, E. Frances, Graduate Student, Yale University.
Clark, Martha L., Research Assistant, Columbia University.
Coldwell, E. Inez, Assistant Professor, North Carolina College for Women.
Cole, Elbert C., Teaching Fellow, Harvard University.
Dellinger, Samuel C., Professor of Zoölogy, University of Arkansas.
Dowden, Florence, Research Student, Yale University.
Emmart, Emily W., Graduate Student, Johns Hopkins University.
Fry, Henry J., Instructor, New York University.
Gowanlock, James N., Associate Professor of Zoölogy, Wabash College.
Hopkins, Aubrey E., Harvard University.
Huntsinger, Harriet C., Research Assistant, Carnegie Institution, Cold Spring Harbor.
Jamieson, M. MacGregor, American Museum of Natural History.
Kahn, Morton C., Instructor in Hygiene, Cornell University Medical College.
Lackey, James B., Assistant in Zoölogy, Columbia University.
Langworthy, Orthello R., Instructor in Anatomy, Johns Hopkins University.
Larmon, Mina L., Research Student, Columbia University.
Lowther, Florence deL., Instructor in Zoölogy, Barnard College.
MacDougall, Mary S., Assistant, Columbia University.
May, Raoul M., Graduate Student, Harvard University.
Moses, Mildred, Research Assistant, Carnegie Institution, Cold Spring Harbor.
Myers, Everett C., Assistant, Johns Hopkins University.
Spencer, Hope, Technician, Yale University.
Springer, Mary G., Instructor in Zoölogy, Columbia University.
Strauss, Maurice B., Student, Amherst College.
Sturtevant, Phoebe R., Carnegie Institution of Washington.
Tharaldsen, Conrad E., Assistant Professor of Zoölogy, Northwestern University.
Thatcher, Lloyd E., Instructor in Zoölogy, University of Michigan.
Thillayampalam, E. M., Research Student, Columbia University.
Wallace, Edith M., Carnegie Institution of Washington.

Warren, D. C., Columbia University.
Whiting, Mrs. Phineas W., Iowa City, Iowa.
Willis, Josephine, University of Pennsylvania.
Wood, Frank E., Professor of Biology, Illinois Wesleyan University.
Woodbridge, Helen, Instructor, University of Maine.
Yolton, Leroy W., Instructor in Biology, Carleton College.
Young, W. C., Amherst College.

INDEPENDENT INVESTIGATORS—Physiology.

Amberson, William R., Assistant Professor, University of Pennsylvania.
Bodansky, Meyer, Adjunct Professor, University of Texas.
Cohn, Edwin J., Assistant Professor of Physiological Chemistry, Harvard University Medical School.
Clowes, George H. A., Director of Research, Eli Lilly & Co., Indianapolis.
Edwards, Dayton J., Associate Professor of Physiology, Cornell University Medical College.
Garrey, Walter E., Professor of Physiology, Tulane University.
Hecht, Selig, Research Fellow, Harvard University.
Hopkins, Hoyt S., Assistant Professor of Physiology, Baylor University Medical College.
Hyde, Ida H., Lawrence, Kansas.
Irwin, Marian, Radcliffe College.
Jacobs, Merkel H., Professor of General Physiology, University of Pennsylvania.
Knowlton, Frank P., Professor of Physiology, Syracuse University.
Lillie, Ralph S., Biologist, Nela Research Laboratories, Cleveland, Ohio.
Loeb, Jacques, Head of Division of Experimental Biology, Rockefeller Institute for Medical Research.
Loeb, Leo, Professor of Comparative Pathology, Washington University.
Morrill, Charles V., Associate Professor of Anatomy, Cornell University Medical College.
Olmsted, James M. D., Associate Professor of Physiology, University of Toronto.
Pond, Samuel E., Physiologist, Nela Research Laboratories, Cleveland, Ohio.
Rapport, David, Cornell University Medical College.
Rapport, Anne Y., Associate in Physiology and Biochemistry, Bryn Mawr College.
Smith, Homer W., Eli Lilly & Co., Indianapolis, Ind.
Spaeth, Reynold A., Associate Professor of Physiology, Johns Hopkins University.
Spirodonovich, R., Instructor, Belgrade Medical University, Belgrade, Servia.

BEGINNING INVESTIGATORS—Physiology.

Blanchard, Kenneth C., Instructor in Chemistry, Massachusetts Institute of Technology.
Cattell, Ware, Assistant, Nela Research Laboratories, Cleveland, Ohio.
Gilson, Arthur S., Jr., Instructor in Physiology, Tulane University.
Hendry, Jessie L., Chemical Assistant, Harvard University Medical School.
Studebaker, Mabel T., Laboratory Technician, Eli Lilly & Co., Indianapolis.

INDEPENDEN INVESTIGATORS—Botany.

Blodgett, Frederick H., Instructor in General Science and Agriculture, State Normal School, Danbury, Conn.
Cleland, Ralph E., Assistant Professor of Biology, Goucher College.
Harper, Robert A., Professor of Botany, Columbia University.

Hazen, Tracy E., Assistant Professor of Botany, Barnard College.
Inman, Ondess L., Head of the Department of Biology, Antioch College.
Lewis, Ivey F., Professor of Biology, University of Virginia.
Lyman, George R., Dean, College of Agriculture, West Virginia University.
Schramm, J. R., National Research Council, Washington, D. C.
Starr, Anna M., Associate Professor of Botany, Mount Holyoke College.
Stevens, Neil E., Pathologist, Department of Agriculture, Washington, D. C.
Stokey, Alma G., Professor of Botany, Mount Holyoke College.
Taylor, William R., Assistant Professor of Botany, University of Pennsylvania.
Weston, William H., Jr., Assistant Professor of Botany, Harvard University.

BEGINNING INVESTIGATORS—Botany.

Blinks, Lawrence R., Harvard University.
Cook, Sherburne F., Graduate Student, Harvard University.
Kemp, Margaret, Radcliffe College.
Wilcox, Marguerite S., Department of Agriculture, Washington, D. C.

STUDENTS.

1923.

Zoology.

Aycock, Robert E., St. Mary's Seminary.
Baker, Herbert N., Instructor, Guilford College.
Bennington, Cecil O., Student, Wabash College.
Blass, Alice M., Student, Carnegie Institute of Technology.
Boyden, Alan A., Assistant in Zoölogy, University of Wisconsin.
Bryngelsson, Lennart G., Student, Knox College.
Byers, Charles F., Student, University of Michigan.
Cattell, Jack, Student, University of Arizona.
Chase, Elton F., Instructor, Tufts College.
Clapp, Eunice E., Student, Smith College.
Cline, Jessie, Student, Mount Holyoke College.
Codding, Mildred B., Student, Wellesley College.
Corey, Edward L., Assistant Instructor in Biology, Western Reserve University.
Cowperthwaite, Marion H., Assistant, Washington University.
Curwen, Alice O., Student, Smith College.
Dodds, Mary L., H. Sophie Newcomb College.
Dowling, Alexander S., Student, De Pauw University.
Eickmann, Reintrant W., Hunter College.
Farrell, Elizabeth G., Student, Notre Dame College of Maryland.
FitzGerald, Eileen M., Student, Connecticut College.
Fogg, Lloyd C., Graduate Student, Dartmouth College.
Freeman, Hazel L., Illinois Wesleyan University.
Fritzell, Agnes I., Student, Connecticut College.
Graves, Clara E., Student, Goucher College.
Harper, Francis D., University of Chicago.
Heilig, Mary D., Student, Carnegie Institute of Technology.
Hill, Benjamin H., Instructor in Biology, Texas Christian University.
Hook, Sabra J., Assistant, University of Rochester.

INDORF, CATHERINE, University of Missouri.
JOHNSON, MARY H., Student, Knox College.
JOHNSON, PERCY L., Student, University of Maine.
KAY, MARY E., H. Sophie Newcomb College.
KOEHRING, VERA, Butler College.
LE COUNT, VERA, University of Cincinnati.
LEONARD, DONALD W., Assistant, Wesleyan University.
LOOMIS, CORNELIA S., Student, Barnard College.
MCMULLEN, ELEANOR C., Instructor in General Zoölogy, Cornell University.
MOORE, IMOGENE, Student, Goucher College.
MORAN, AGNES E., Student, University of Rochester.
MORGAN, WILLIAM O., Columbia University.
MORRIS, ALDEN W., Student, DePauw University.
MORTENSEN, EDITH E., Student, Carleton College.
MOSSMAN, HARLAND W., Assistant in Zoölogy, University of Wisconsin.
MULHERN, GRACE W., Student, Notre Dame College of Maryland.
NESTER, HENRY G., Student, Butler College.
OSTERTAG, BERNARD, Student, College for Women, Western Reserve University.
PICKERING, ELEANOR C., Student, Oberlin College.
PIERIS, PAUL E., Student, Trinity College, Cambridge, England.
RICHARDSON, DOROTHY, Student, Mount Holyoke College.
SANDERS, ELIZABETH P., Student, Goucher College.
SEVRENS, OLIVER F., Principal, Philippine Academy.
SOBEL, MOLLIE, Student, Hunter College.
SPALDING, JANET E., Elmira College.
SPILMAN, ROBERT S., Post Surgeon and Professor of Biology, Virginia Military Institute.
TOBIAS, CLARENCE E., JR., Student, University of Pennsylvania.
WARBASSE, AGNES, Packer Collegiate Institute.
WILLIAMS, ELISABETH, Student, Mount Holyoke College.
WILLIAMS, MERRITT F., Student, Washington University.
WINKLEY, RUTH, Student, University of Michigan.

Embryology.

BAUMAN, BEN, Instructor, De Witt Clinton High School, New York City.
BEERS, CHARLES D., Student, Johns Hopkins University.
BOOTH, ESTHER, Elmira College.
BOWEN, EDITH S., Assistant in Biology, Brown University.
BUEHLER, EUGENE O., Student, Wabash College.
CAPPS, PRISCILLA, Smith College.
DAVIS, SARAH H., Radcliffe College.
DIXON, PHYLLIS, Vassar College.
GRAY, IRVING E., Assistant, University of Wisconsin.
JOHNSON, MAYNARD S., Graduate Student, University of Illinois.
JOHNSON, WILLIS H., Student, Wabash College.
JONES, MYRNA F., Student, Doane College.
JULIN, MARY, Zurich, Switzerland.
KEEFE, ANSELM M., St. Norbert's College.
KESSLER, CLIFTON P., Student, Wabash College.
KUBICEK, HELEN M., Student, Doane College.
LEE, MILTON O., Instructor, Ohio State University.

Mandeville, Mary, Elmira College.
Marshall, Eleanor H., Women's College of University of Delaware.
Millemann, R. J., Student, Cornell University Medical College.
Montague, Ouida, Shrewsbury, Mass.
Moss, Leland C., Student, Wabash College.
Murray, Margaret R., Assistant, Washington University.
Parsons, Talcott, Student, Amherst College.
Perry, Lydia S., Assistant, Mount Holyoke College.
Pike, Arlene H., Saugus, Mass.
Turner, Winifred H., Student, Smith College.
Warner, William R., Student, Carleton College.
Watt, Lucy J., Instructor, Wells College.
Whaley, James H., Jr., Cornell University Medical College.
Williams, Dorothy E., University of Wisconsin.

Physiology.

Banning, Laura, Teacher, Morris High School, Bronx, New York City.
De Marco, Carlo M., Jr., Student, University of Pennsylvania.
Edsall, John T., Harvard University.
Egloff, William C., Student, University of Chicago.
Emerson, Robert, Student, Harvard University.
Espenscheid, Gertrude K., 617 Third St., Brooklyn, N. Y.
Florence, Loree, Technician, Medical Dept., University of Georgia.
Harrison, William G., Jr., Student, University of Michigan.
Hartline, Haldan K., Student, Lafayette College.
Hitchcock, Fred A., Graduate Student, Ohio State University.
Keeler, Romola L., 264 West 95th St., New York City.
Lucas, Alfred M., Student, Wabash College.
Lucke, Baldwin, Assistant Professor of Pathology, University of Pennsylvania.
McCutcheon, Morton, Instructor in Pathology, University of Pennsylvania.
Mayerson, Hymen S., Assistant in Physiology, Yale University.
Plunkett, Charles R., 1603 Newkirk Ave., Brooklyn, N. Y.
Ring, Gordon C., Assistant, Wesleyan University.
Schmitt, Francis O., Student, Washington University.
Stedman, Hazeltene L., Instructor in Physiology, Mount Holyoke College.
Stephens, Mary A., Student, Elmira College.
Turner, Abby H., Professor of Physiology, Mount Holyoke College.
Worcester, Blandina, Radcliffe College.

Attending Morning Lectures Only.

Ashby, Winifred M., The Mayo Foundation, Rochester, Minn.
Bertsch, Marguerite, 838 Riverside Drive, New York City.
Goldberg, Jacob A., 356 Second Ave., New York City.
Halsey, John T., 1232 Maison Blanche Building, New Orleans.
Pertzoff, V. S., 25 Hillside Ave., Cambridge, Mass.

Protozoölogy.

Bond, Lester E., Associate Professor of Biology, Maryville College.
Burdick, Donald L., Instructor in Biology, New York University.
Cook, Daniel, University of Cincinnati.
Dailey, William T., Instructor, New York University.

Darby, Hugh H., Student, Columbia University.
Dimick, Gertrude P., Graduate Student, Brown University.
Greenberg, Frances, Columbia University.
Griffin, Donald C., Instructor in Biology, New York University.
Hauber, Ulrich A., St. Ambrose College.
Haupt, George W., Bucknell University.
Hitchens, A. Parker, Instructor in Bacteriology, Army Medical School.
Knapke, Bede, St. Bernard College.
O'Toole, George B., Professor of Theology and Animal Biology, St. Vincent Archabbey.
Ruckes, Herbert, Instructor, College of the City of New York.
Webster, William D., Instructor, New York University.
Wenstrup, Edward J., St. Vincent Archabbey.

Botany.

Acquarone, Paul, Student, Pennsylvania State College.
Brown, Alice E., Student, Goucher College.
Brown, Annette, Student, Sweet Briar College.
Castle, Edward S., Student, Harvard University.
Clarke, Alice E., Student, Goucher College.
Curran, Loretta J., Teacher, Boston, Mass.
Duckworth, Helen E. A., Student, Sweet Briar College.
Fogg, John M., Jr., University of Pennsylvania.
Greig, Mary, American Museum of Natural History.
Hof, Anne C., Student, George Washington University.
Jamieson, Janet P., Teacher, West Philadelphia Girls' High School.
Mackintosh, Elizabeth L., Student, Smith College.
Nute, Bertha E., Science Teacher, Hastings-on-Hudson.
Overstreet, Mrs. Harry A., 98 Morningside Ave., New York City.
Pike, Radcliffe B., Student, Bowdoin College.
Shaw, Margaret F., Instructor, Vassar College.
Stiffler, Ethel G., Student, Goucher College.
Sumwalt, Margaret, Student, Goucher College.

6. TABULAR VIEW OF ATTENDANCE.

	1919	1920	1921	1922	1923
INVESTIGATORS—Total	134	136	172	182	176
Independent:					
Zoölogy	68	69	79	87	90
Physiology	24	22	26	28	23
Botany	7	7	13	15	13
Under Instruction:					
Zoölogy	21	29	34	34	41
Physiology	10	7	11	11	5
Botany	4	2	9	7	4

Students—Total	128	120	120	126	146
Zoölogy	55	56	53	59	59
Protozoölogy	15	15	11	12	16
Embryology	33	26	28	28	31
Physiology	17	15	18	19	22
Botany	8	8	10	8	18
Total Attendance	262	256	292	308	322
Institutions Represented—Total	88	86	95	104	107
By investigators	61	55	67	71	62
By students	62	57	53	68	73
Schools and Academies Represented.					
By investigators	–	1	–	–	–
By students	4	7	–	–	4

7. SUBSCRIBING AND COÖPERATING INSTITUTIONS.

A coöperating institution is one that has subscribed for the two preceding years, or that announces its intention of subscribing regularly. A subscribing institution is one that pays for one or more tables or rooms.

SCHOLARSHIP TABLES

Lucretia Crocker Scholarships for Teachers in Boston.

Scholarship of $100.00 Supported by a friend of the Laboratory since 1898.

The New London Branch of the American Association of University Women, since 1920.

SUBSCRIBING AND COÖPERATING INSTITUTIONS IN 1923

American Museum of Natural History
Amherst College
Antioch College
Barnard College
Bowdoin College
Bryn Mawr College
Butler College
Carleton College
Carnegie Institute of Technology
Carnegie Institution of Washington, Dept. of Embryology
Carnegie Institution, Cold Spring Harbor
College of Physicians and Surgeons
Columbia University
Connecticut College
Cornell University
Cornell University Medical College
Dartmouth College
De Pauw University
Doane College

Elmira College
Goucher College
Harvard University
Harvard University Medical School
Hunter College
Illinois Wesleyan University
Johns Hopkins University
Johns Hopkins University Medical School
Knox College
Lafayette College
Eli Lilly & Co.
Mount Holyoke College
Nela Research Laboratories
New York University
North Carolina College for Women
Northwestern University
Notre Dame College
Oberlin College
Princeton University
Radcliffe College
Rockefeller Foundation
Rockefeller Institute for Medical Research
Smith College
Sophie Newcomb College
St. Bernard College
St. Mary's Seminary
St. Norbert's College
St. Vincent Archabbey
Sweet Briar College
Tufts College
Union College
U. S. Army Medical Department
U. S. Veterans Bureau
University of Arkansas
University of Chicago
University of Cincinnati
University of Illinois
University of Maine
University of Michigan
University of Minnesota
University of Missouri
University of Pennsylvania
University of Rochester
University of Wisconsin
Vassar College
Wabash College
Washington University
Washington University Medical School
Wellesley College
Wesleyan University
Western Reserve University
Wistar Institute of Anatomy and Biology
Yale University

8. EVENING LECTURES, 1923.

Friday, July 6,
Dr. Edwin B. Wilson.......... "Notes on Statistical Methods."

Tuesday, July 10,
Dr. A. F. Blakeslee........... "Genetics of the Jimson Weed."

Friday, July 13,
Dr. C. M. Child............... "The Physiological Gradients as Factors in Development."

Monday, July 16,
Dr. Tracey E. Hazen.......... "Botanizing in the Colombian Andes."

Tuesday, July 17,
Dr. E. V. Cowdry.............. "The Physiological Significance of the Golgi Apparatus in the Cells of Animals and Plants."

Friday, July 20,	Dr. A. H. Sturtevant	"Mutation."
Tuesday, July 24,	Dr. Edward C. Schneider	"The Reaction of the Human Body to Variations in Atmospheric Oxygen."
Tuesday, July 31,	Mr. William Lyman Underwood	"Journeys with a Passamaquoit Indian."
Friday, Aug. 3,	Dr. R. W. Hegner	"Some Investigations on Entozoic Protozoa."
Tuesday, Aug. 7,	Dr. C. C. Little	"The Occurrence of Heritable Abnormalities in Mice and their Relation to Exposure to X-Rays."
Friday, Aug. 10,	Dr. S. R. Detwiler	"Embryonic Surgery in Relation to Problems of Development in the Nervous System."
Tuesday, Aug. 14,	Dr. J. A. Detlefsen	"Habituation and Dishabituation."

9. MEMBERS OF THE CORPORATION.

1. Life Members.

Allis, Mr. E. P., Jr., Palais Carnoles, Menton, France.
Andrews, Mrs. Gwendolen Foulke, Baltimore, Md.
Billings, Mr. R. C., 66 Franklin St., Boston, Mass.
Carey, Mr. Arthur Astor, Fayerweather St., Boston, Mass.
Clarke, Prof. S. F., Williamstown, Mass.
Conklin, Prof. Edwin G., Princeton University, Princeton, N. J.
Crane, Mr. C. R., Woods Hole, Mass.
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Foot, Miss Katherine, Care of Morgan Harjes Cie, Paris, France.

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Gardiner, Miss Eugenia, 15 W. Cedar St., Boston, Mass.
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Jackson, Mr. Chas. C., 24 Congress St., Boston, Mass.
Kidder, Mr. Nathaniel T., Milton, Mass.
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Minns, Mr. Thomas, 14 Louisburg Square, Boston, Mass.
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Morgan, Mrs. T. H., New York City, N. Y.
Noyes, Miss Eva J.
Nunn, Mr. Lucian L., Telluride, Colo.
Osborn, Prof. Henry F., American Museum of Natural History, New York City, N. Y.
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Phillips, Mrs. John C., Windy Knob, Wenham, Mass.
Porter, Dr. H. C., University of Pennsylvania, Philadelphia, Pa.
Pulsifer, Mr. W. H., Newton Center, Mass.
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2. REGULAR MEMBERS, AUGUST, 1923.

ADAMS, MISS A. E., Mount Holyoke College, South Hadley, Mass.
ADDISON, DR. W. H. F., University of Pennsylvania Medical School, Philadelphia, Pa.
ADOLPH, DR. EDWARD F., University of Pittsburgh, Pittsburgh, Pa.
AGERSBORG, DR. H. P. K., Williams College, Williamstown, Mass.
ALLEE, DR. W. C., University of Chicago, Chicago, Ill.
ALLEN, PROF. EZRA, Ursinus College, Collegeville, Pa.
ALLYN, MISS HARRIET M., Hackett Medical College, Canton, China.
ALTENBURG, DR. EDGAR, Rice Institute, Houston, Texas.
AMBERSON, DR. WILLIAM B., University of Pennsylvania, Philadelphia, Pa.
ANDERSON, DR. E. G., University of Michigan, Ann Arbor, Mich.
ATTERBURY, MRS. RUTH R., College of Physicians and Surgeons, New York City.
BAITSELL, DR. GEORGE A., Yale University, New Haven, Conn.
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BAKER, DR. E. H., Cooper Carlton Hotel, Hyde Park Station, Chicago, Ill.
BALDWIN, DR. F. M., Iowa State College, Ames, Iowa.
BASCOM, DR. K. F., University of Pennsylvania, Philadelphia, Pa.
BECKWITH, DR. CORA J., Vassar College, Poughkeepsie, N. Y.
BEHRE, DR. ELINOR H., Louisiana State University, Baton Rouge, La.
BIGELOW, PROF. M. A., Teachers College, Columbia University, New York City.
BIGELOW, PROF. R. P., Massachusetts Institute of Technology, Cambridge, Mass
BINFORD, PROF. RAYMOND, Guilford College, Guilford College, N. C.

Blodgett, Dr. F. H., State Normal School, Danbury, Conn.

Bodansky, Dr. Meyer, University of Texas, Galveston, Texas.

Boring, Dr. Alice M., Yenching College, Peking, China.

Box, Miss Cora May, University of Cincinnati, Cincinnati, Ohio.

Bowen, Dr. Robert H., Columbia University, New York City.

Bradley, Prof. Harold C., University of Wisconsin, Madison, Wis.

Brailey, Miss Miriam E., Mount Holyoke College, South Hadley, Mass.

Bridges, Dr. Calvin B., Columbia University, New York City.

Brooks, Dr. S. C., U. S. Public Health Service, Washington, D. C.

Brumfiel, Dr. Daniel M., Johns Hopkins University Medical School, Baltimore, Md.

Buckingham, Miss Edith N., 342 Marlboro St., Boston, Mass.

Budington, Prof. R. A., Oberlin College, Oberlin, Ohio.

Bumpus, Prof. H. C., Brown University, Providence, R. I.

Byrnes, Dr. Esther F., 193 Jefferson Ave., Brooklyn, N. Y.

Calkins, Prof. Gary N., Columbia University, New York City.

Calvert, Prof. Philip P., University of Pennsylvania, Philadelphia, Pa.

Carlson, Prof, A. J., University of Chicago, Chicago, Ill.

Carothers, Dr. Eleanor E., University of Pennsylvania, Philadelphia, Pa.

Carroll, Prof. Mitchel, Franklin and Marshall College, Lancaster, Pa.

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Casey, Colonel Thomas L., Washington, D. C.

Casteel, Dr. D. B., University of Texas, Austin, Texas.

Cattell, Prof. J. McKeen, Garrison-on-Hudson, N. Y.

Cattell, Mr. McKeen, Harvard Medical School, Boston, Mass.

Chambers, Dr. Robert, Cornell University Medical College, New York City.

Charlton, Dr. Harry H., University of Missouri, Columbia, Mo.

Chidester, Prof. F. E., West Virginia University, Morgantown, W. Va.

Child, Prof. C. M., University of Chicago, Chicago, Ill.

CLAPP, PROF. CORNELIA M., Mount Holyoke College, South Hadley, Mass.

CLARK, PROF, E. R., University of Georgia, Augusta, Ga.

CLOWES, PROF. G. H. A., Eli Lilly & Co., Indianapolis, Ind.

COE, PROF. W. R., Yale University, New Haven, Conn.

COHN, DR. EDWIN J., Harvard Medical School, Boston, Mass.

COKER, DR. R. E., University of North Carolina, Chapel Hill, No. Carolina.

COLE, DR. LEON J., College of Agriculture, Madison, Wis.

COLLETT, DR. MARY E., Tulane University, New Orleans, La.

COLLEY, MRS. MARY W., 1712 Madison St., Madison, Wis.

COLTON, PROF. H. S., Ardmore, Pa.

COOLIDGE, MR. C. A., Ames Building, Boston, Mass.

COPELAND, PROF. MANTON, Bowdoin College, Brunswick, Maine.

COWDRY, DR. E. V., Rockefeller Institute, New York City.

CRAMPTON, PROF. H. E., Barnard College, Columbia University, New York City.

CRANE, MRS. C. R., Woods Hole, Mass.

CURTIS, PROF. W. C., University of Missouri, Columbia, Mo.

CURTIS, DR. MAYNIE R., Crocker Laboratory, Columbia University, New York City.

DAKCHAKOFF, DR. VERA, College of Physicians and Surgeons, New York City.

DAVIS, DR. DONALD W., College of William and Mary, Williamsburg, Va.

DAVIS, PROF. BRADLEY M., University of Michigan, Ann Arbor, Mich.

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DAWSON, DR. J. A., Harvard University, Cambridge, Mass.

DEDERER, DR. PAULINE H., Connecticut College, New London, Conn.

DEXTER, DR. J. S., Hotel Miramar, Santurce, Porto Rico.

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DONALDSON, DR. JOHN C., University of Pittsburgh, School of Medicine, Pittsburgh, Pa.

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Dunn, Dr. Elizabeth H., Woods Hole, Mass.
Edwards, Dr. D. J., Cornell University Medical College, New York City.
Eigenmann, Prof. C. H., University of Indiana, Bloomington, Ind.
Ellis, Dr. F. W., Monson, Mass.
Farnum, Miss Louise W., Peking, China.
Field, Miss Hazel E., Sophie Newcomb College, New Orleans, La.
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Gage, Prof. S. H., Cornell University, Ithaca, N. Y.
Garrey, Prof. W. E., Tulane University, Richardson Memorial, New Orleans, La.
Gates, Prof. R. Ruggles, University of London, England.
Geiser, Dr. S. W., Washington University, St. Louis, Mo.
Glaser, Prof. O. C., Amherst College, Amherst, Mass.
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HEILBRUNN, DR. L. V., University of Michigan, Ann Arbor, Mich.

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MCGILL, DR. CAROLINE, Murray Hospital, Butte, Montana.
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MCINDOO, DR. N. E., Bureau of Entomology, Washington, D. C.
MCMURRICH, PROF. J. P., University of Toronto, Canada.
MCNAIR, DR. G. T., 1721 Grand Ave., Chickasha, Okla.
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MALONE, PROF. E. F., University of Cincinnati, Cincinnati, Ohio.
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MARTIN, MR. E. A., College of the City of New York, New York City.
MAST, PROF. S. O., Johns Hopkins University, Baltimore, Md.
MATHEWS, PROF. A. P., University of Cincinnati, Cincinnati, Ohio.
MATSUI, PROF. K., Imperial College of Agriculture and Dendrology, Morioka, Japan.
MAVOR, PROF. JAMES W., Union College, Schenectady, N. Y.
MEDES, DR. GRACE, Wellesley College, Wellesley, Mass.

---

[1] Deceased.

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Miner, Dr. Roy W., American Museum of Natural History, New York City.

Mitchell, Dr. Philip H., Brown University, Providence, R. I.

Moore, Prof. George T., Missouri Botanical Garden, St. Louis, Mo.

Moore, Dr. Carl R., University of Chicago, Chicago, Ill.

Moore, Prof. J. Percy, University of Pennsylvania, Philadelphia, Pa.

Moore, Dr. A. R., Rutgers College, New Brunswick, N. J.

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Packard, Dr. W. H., Bradley Polytechnic Institute, Peoria, Ill.

Pappenheimer, Dr. A. M., Columbia University, New York City.

Parker, Prof. G. H., Harvard University, Cambridge, Mass.

Paton, Prof. Stewart, Princeton University, Princeton, N. J.

Patten, Prof. William, Dartmouth College, Hanover, N. H.

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Phillips, Dr. E. F., Department of Agriculture, Washington, D. C.

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Reinke, Dr. E. E., Vanderbilt University, Nashville, Tenn.

Rice, Prof. Edward L., Ohio Wesleyan University, Delaware, Ohio.

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Richards, Mrs. A., Norman, Okla.

Rhodes, Prof. Robert C., Emory University, Atlanta, Ga.

Robertson, Prof. W. R. B., University of Kansas, Lawrence, Kansas.

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Romer, Dr. Alfred S., University of Chicago.

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BIOLOGICAL BULLETIN

MISCELLANEOUS BIOLOGICAL OBSERVATIONS ON BUMBLEBEES.[1]

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Although the biology of bumblebees has been a favorite study of numerous amateur and professional naturalists, many questions concerning the life-history and habits of these interesting insects are still unsolved. In several recent papers (1922 *a*, *b*, *c*; 1923 *a*, *b*), I have discussed some of these questions, and in the following paragraphs wish to touch briefly upon several others which, because of their heterogeneity, could not be incorporated in the earlier papers.

I. Condition of Pile and Nesting Habit.

In his monograph on the bumblebees of Thuringia, Schmiedeknecht (1878) expresses the belief that loss of thoracic pile in bumblebees indicates that the colonies to which such individuals belong have subterranean nests. That this theory is incorrect is shown by the following observation. On July 6, 1921, I transferred a surface nest of *Bremus* (*Bombus*) *fervidus* to an observation-box in which the colony was kept until the first part of October. At the time the nest was taken, the pile of all the members of the colony, including that of the queen, was in perfect condition, and remained that way until the latter part of July, when the dorsal side of all the foraging workers became coated with a layer of yellow pollen. These layers of pollen gradually hardened and sometimes reached several millimeters in thickness. The bees evidently were annoyed by these lumps of pollen, for they repeatedly tried to rub them off with their front legs. Whenever they succeeded in doing so, most of the pile on the dorsal side of the thorax came off with the pollen,

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[1] Contributions from the Entomological Laboratory of the Bussey Institution, Harvard University. No. 226.

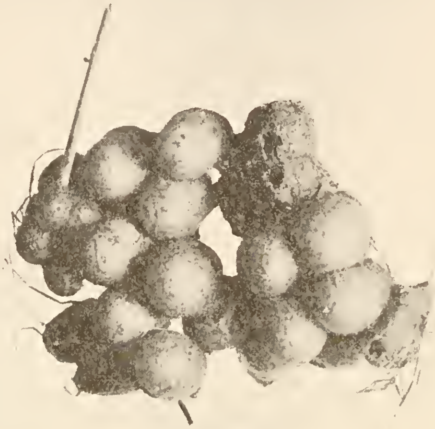

Fig. 1

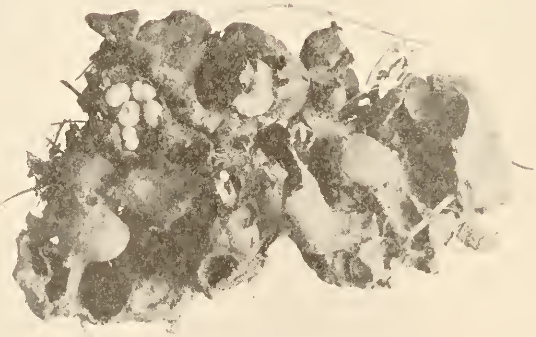

Fig. 2

FIG. 1. Comb and brood produced by a queenless colony of *Bremus fervidus* Fabricius.

FIG. 2. Comb and brood produced by a queenless colony of *Bremus impatiens* Cresson.

leaving the center of the mesonotum completely bare. Similar observations were made on several *Bremus fervidus* colonies during the summer of 1922. Investigation showed that these lumps of pollen were acquired by the bees while they were gathering nectar from *Linaria*, popularly known as butter and eggs, or toad flax. In the light of these facts, it is not surprising that Schmiedeknecht's (pp. 384, 424) predictions in regard to the nesting habits of some of the rarer European species have proved to be incorrect (cf. Gundermann, 1908, p. 31). This is also true of some of the predictions of Hoffer (cf. Härter, 1890, pp. 71–73).

II. The Structure of the Outer Nest.

Wagner (1907) states that the fibrous envelope of epigeic bumblebee nests is composed of two distinct layers which he calls the outer and the inner nest. Although I have examined a large number of epigeic nests belonging to several American and European species, I have never found an indication of such an arrangement, and this leads me to believe that such nests as those shown by Wagner (pp. 31–32) in Figs. 14, 15, and 16, instead of being of universal occurrence, are extremely rare.

III. The Population of a Bumblebee Colony at Night.

Packard (1865), Coville (1890), and Sladen (1912) state that the whole population of a bumblebee colony is at home during the night, while Shuckard (1866), whose observations agree with those of Hoffer (1882/83), has the following to say concerning this matter: "If, in their collecting excursions, they are intercepted by heavy rains, or loiter far away too long until twilight closes, they will pass the night away from home, and return laden with their gatherings as soon as the warmth of the sun reanimates them to activity; thus they will often sleep in flowers, and a nest therefore taken at night is not always a sure indication in those found within it, of its complete population." For many years, I also held the same opinion as Packard (p. 109), Coville (p. 198), and Sladen (pp. 47, 99), but during the summer of 1921, I accidentally discovered that the observations of Shuckard (p. 315) and Hoffer (p. 5) are correct.

IV. Size of Colony and Nest Cavity.

Wagner (1907, p. 36) states that the size of the nest cavity of species which nest in the ground corresponds to the size of the colony. In reply to this assertion, I may state that during the summers of 1921 and 1922 I dug up a number of incipient bumblebee colonies whose nests filled less than half of the nest cavity.

Although he has never actually observed this, it is further claimed by Wagner (pp. 36, 153–154) that bumblebees excavate, or at least enlarge, subterranean cavities for their nests. Sladen (1912, p. 40), on the other hand, has the following to say concerning this question: "As the larger and newer cocoons become available for storage of food, the oldest ones at the bottom of the comb are emptied and used no more, except in a time of plenty, when all the rest are full. In underground nests, where all available space is likely to be needed for the expansion of the comb, the walls of these abandoned cocoons are often bitten down, and the comb sinks. Wasps, it is well known, enlarge their nest cavity according to their requirements by digging out little lumps of earth and flying away with them, but I have never seen bumblebees do this." To this statement, which agrees with my own observations, I may add that the fragments of such dismantled cocoons are sometimes found intermingled with the material, *e.g.* dry grass, which with the bees cover the comb.

V. The Proximity of Bumblebee Colonies.

Some years ago, Wagner (1907) discovered a colony of *Bremus muscorum*, and about 35 cm. from it a destroyed bumblebee nest. This led him to conclude that the members of the colony had left the old nest and had built a new one, either because their larvae had been destroyed by parasites or because they had been robbed of their comb, for bumblebee colonies, according to Wagner (p. 157), never nest in such close proximity. In reply to this assertion of Wagner (1907), I may state that I recently (June 26, 1922) discovered a colony of *Bremus impatiens* and one of *Bremus vagans* which had their nests within two feet of each other, and, judging from my experience with colonies in artificial nests, there seems to be no reason why bumblebees could not nest in even closer proximity.[1]

[1] On July 29, 1923, shortly after the manuscript of this paper had been mailed

VI. The Defense of the Nest.

Wagner (1907) claims that one, or a small number of bumblebees, even if they belong to a populous colony, never attack, and that the courage of a bumblebee depends upon the number of individuals present. This certainly is not true of species like *Bremus fervidus*, *impatiens*, *lapidarius*, and *terrestris*. On more than one occasion I have been attacked with great ferocity by single workers of these species at the old nest-site, while the remaining members of the colony were miles away. Equally incorrect, as Hoffer (1888, pp. 97–98), Sladen (1912, pp, 254, 277), and I (1922 *a*, p. 34) have shown, is Wagner's claim (pp. 144–145) that the members of a bumblebee colony do not coöperate in expelling strange bumblebees from their nest.

As can easily be demonstrated, the temper of a bumblebee colony depends upon a number of factors. If, for instance, the colony is chilled, even savage species, *e.g.* *Bremus fervidus*, exhibit little or no fighting spirit. This lack of temper becomes still more marked if, in addition, the colony has been compelled to fast. The ferocity of a bumblebee colony, to a certain extent, also depends upon previous experience. If the nest of a savage species, like *Bremus fervidus*, is frequently disturbed, certain members of the colony will attack any one whom they find in the vicinity of their nest. It is probable, therefore, that Wagner (pp. 151–152), at least in the case of *Bremus lapidarius* and *terrestris*, based his conclusions on observations which were made at a time when the mettle of his colonies was low as a result of adverse conditions.

VII. Incubation of the Brood.

There is a difference of opinion among bumblebee students as to why bumblebees incubate their brood. Hoffer (1882/83, I, p. 14) has the following to say concerning this activity: "Die Königin liegt dabei häufig so über der Zelle, dass sie dieselbe mit dem Bauche wärmt, wie eine Henne ihre Eier, wobei der Bauch dicht an die Zelle angedrückt wird. Dieses Bebrüten übt sie übrigens auch an älteren Eier- und Larvenklumpen und den Puppentönnchen aus." A similar view is held by Sladen (1912,

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to the editor, I took two flourishing bumblebee colonies—one of *Bremus fervidus* and the other of *Bremus pennsylvanicus*—which had their nests within a foot of each other.

pp. 25–31). However, this explanation is rejected by von Buttel-Reepen (1903), who suggests that, on the contrary, bumblebees, being guided solely by pleasant stimuli in such cases, probably incubate their brood because they profit by the warmth which is given off by the larvæ. Several well-founded objections to von Buttel-Reepen's (pp. 38–39) theory have been published by Wagner (1907). Among other things, Wagner (pp. 93–95) has pointed out that bumblebees incubate objects from which they cannot possibly get warmth, a statement which is corroborated by my own observations. Thus, for example, I have frequently seen queens and workers of various species incubate the outer rim of tin cans which were used in artificial breeding experiments, even when the thermometer was below the optimum temperature for bumblebees. While external stimuli undoubtedly play an important part in this interesting habit of bumblebees, broodiness probably also depends upon a certain physiological condition. Only in this way, it seems to me, can one explain the fact that it sometimes takes several weeks until a confined *Bremus* queen becomes broody, and that, after becoming so, she readily incubates various objects, such as tin cans or the floor and sides of her nest-box, in some cases even days or weeks before she lays her first eggs.

VIII. Red Clover and Pollen.

In his monograph on bumblebees referred to at the beginning of this paper, Schmiedeknecht (p. 328) claims that bumblebees cannot obtain pollen from clover. This statement likewise is incorrect. The *corbiculæ* of bumblebees working on red clover almost always contain dark-brown pollen, and by a half hour's observation one can easily convince oneself that this pollen is obtained from the clover.

IX. Method of Depositing Pollen.

Another point on which there is a difference of opinion, is the question as to how a bumblebee deposits her load of pollen. Coville (1890), whose observations agree with those of Sladen (1912), describes this activity as follows: "If pollen-laden, the bee balanced herself, with her middle and cephalic pairs of legs, on the edge of a pollen-pot, head outward, spread her wings, and then scraped the pollen-masses from her *corbiculæ* by rubbing the posterior legs together." Hoffer (1882/83, I, p. 16), Wagner (1907, p. 98), and Frison (1917), on the other hand, state that the bumblebee removes the pollen with the middle pair of legs, and this agrees with my own observations. Frison (pp. 283–284) describes the process as follows: "After the pollen-containing cocoon has been found, the worker stands on the edge facing away from the cocoon, and inserts her hind legs down into the cocoon; then she proceeds by a quick, slicing downward movement of the middle pair of legs, to remove the pollen from the *corbicula*." It is difficult to conceive how bumblebees could deposit pollen in the manner described by Coville (p. 200), and Sladen (p. 46), and it seems probable therefore that these two authors overlooked the rapid movement of the second pair of legs.

X. On the Behavior of Pollen-laden Bumblebees in Late Fall.

Wagner (1907) claims that bumblebees suffer a great reduction of instincts toward the end of the breeding season. As one of the proofs for this assertion, he states that in late fall pollen-laden bumblebees return to the nest, but leave again without depositing their pollen. Although I had about 75 bumblebee colonies under observation during the summers of 1921 and 1922, I was unable to observe any such behavior, and this leads me to believe that there were other reasons than those given by Wagner (p. 190) why his bees left without depositing their pollen.

XI. The Capping of Honey Cells.

Hoffer (1882) and Sladen (1912), the two foremost European authorities on the biology of bumblebees, differ as to whether bumblebees cap their honey. Hoffer (I, p. 39) has the following to say concerning this question: "Ist das Honigtöpfchen voll, so wird sehr häufig ein stumpf-kegelförmiger Deckel darüber gebaut, der aber in der Mitte eine ganz kleine Oeffnung hat, so dass also niemals vollständig gedeckelte Honigzellen angetroffen werden, wie sie die Bienen haben." Sladen (p. 53), on the other hand, says: "In a favourable season a populous colony may have all the vacated cocoons, amounting to over 400, filled with thick honey and sealed over with wax." Observations made on *Bremus ternarius* (by Putnam, 1865), *Bremus auricomus* (by Frison, 1917), and *Bremus impatiens* (by myself) in this country, confirm those of Sladen (1912).[1] In large colonies of *Bremus impatiens*, I have repeatedly found a considerable portion of the lower cocoons filled with thick honey and completely capped so that no opening could be seen, even with the aid of a hand lens.

XII. The Food of Bumblebee Larvæ.

In his extensive paper on the hive-bee,[2] Swammerdam (1752) also refers incidentally to some of his biological observations on bumblebees, and expresses the belief that the larvæ of the latter feed on the wax by which they are surrounded, since, in his opinion, it seems improbable that the adults open the wax covering to feed the larvæ. But Huber (1802) found that this is precisely what the adults do, and that the food of the larvæ consists of a mixture of pollen and honey, and not of wax. These observations of Huber (pp. 263–265) were later confirmed by Schmiedeknecht (1878, p. 321) and Hoffer (1882/83). However, several years later, Härter (1890), being unable to observe any such method of feeding, questioned the correctness of Hoffer's (p. 27) observations and came out strongly in support of Swammerdam's (pp. 194–195) hypothesis. Since, to my knowledge, Härter's (p. 67) claim has never been denied, I should here like to mention the further positive evidence by Coville (1890) and Sladen (1912) whose observations agree with my own. In this connection, it may be stated that the Pocket-makers, as was first pointed out by Sladen (1899), use a somewhat different method of feeding their larvæ, at least those of the workers. This question will be discussed more fully in connection with another paper.

XIII. Assistance to Emerging Young.

Huber (1802), Dahlbom (1832), and von Buttel-Reepen (1903) state that bumblebees assist their young during the process of hatching by widening the slit which the emerging bee makes in its cocoon. Not long ago, Wagner (1907) subjected this question to the experimental test. He isolated some cocoons of *Bremus lapidarius* and found that the first worker which emerged—although two days old—did not pay the slightest attention to the second when the latter was struggling to leave its cocoon. From this rather superficial experiment, Wagner (p. 108) infers that bumblebees do not assist their young during the process of hatching. However, against this negative evidence of Wagner (1907), we have the further positive evidence of Sladen (1912), Bachmann (1915), and Frison (1917), whose observations I have been able to confirm.

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[1] Since writing the above, I have discovered that the French Abbé Noël de Pluche (1764) also made this observation.

[2] This paper (cf. Swammerdam, p. vi) was completed in 1673.

XIV. The Adoption of Individuals from Other Colonies

Wagner (1907, p. 172) claims that after a bumblebee has been in the nest of another species from 10 to 12 hours she is, without exception, treated as a member of the colony. This statement, as well as Wagner's (p. 172) assertion that the specific odor of a bumblebee colony emanates only from the queen, is disproved by the results of several breeding experiments which I carried out during the summer of 1922 (cf. 1923*a*). These experiments show that in some cases even members of such closely related species as *Bremus fervidus* and *Bremus pennsylvanicus* may refuse to have anything to do with each other, even after they have been together for weeks, and although the members of one of these species have been reared by the other.

XV. The Homing Instinct of Queens and Workers.

Wagner (1907) states that young bumblebee queens take a much longer orientation flight than the workers, and that despite this fact they have much more difficulty in finding the nest than the latter. I have observed the first orientation flight of a large number of young queens and workers belonging to several species, and although I found considerable individual variation in the aptitude which these bees showed in finding the nest, I failed to note any such correlation as that described by Wagner (pp. 218–219). In fact, if any generalizations can be made, I should say that the young queens appeared to have less difficulty in finding the nest than the workers.

XVI. Natural Requeening.

In the works of Hoffer (1882/83), Handlirsch (1888a, 1888b, 1891), Sharp (1899), Friese and Wagner (1904, 1910, 1912, 1914), Vogt (1909, 1911), and Franklin (1912/13), one occasionally meets with the statement that the members of a bumblebee colony are the offspring of one queen, and on this "fact" several of the above-mentioned authors have based far-reaching conclusions in regard to the genetic constitution of certain species. However the observations of Putnam (1865), Lindhard (1912), Sladen (1912), and Frison (1921) indicate that this is by no means always the case, since queens which have started colonies are often killed and supplanted by others which enter their nest, a fact which is corroborated by my own observations (cf.1922*c*, 1923*a*). Here in the vicinity of Boston, this natural requeening is especially common with *Bremus fervidus* and *Bremus impatiens.*

XVII. Nests Established by Queenless Workers.

Wagner (1907) believes that a bumblebee colony which has lost its queen and comb cannot build another nest, and hence is unable to contribute anything toward the next generation. Huber (1802, pp. 288–289), on the other hand, states that bumblebee workers which have lost both queen and comb are able to start a new nest.[1]

In order to determine which one of these two authors is correct, the following experiments were carried out. On August 1, 1922, I removed all the comb from a queenless colony of *Bremus fervidus* which at that time consisted of about 100 workers. The latter at once started to build a new nest and produced a considerable number of males. Two weeks later, the same experiment was performed with a colony of *Bremus impatiens* consisting of about 300 workers, and this yielded the same result. The accompanying plate shows how the comb of the two colonies looked on August 21, 1922. These experiments prove conclusively that Wagner's (pp. 116–117, 154–155) contention is incorrect.

XVIII. The Efficiency of Bumblebees.

Wagner (1907) claims that bumblebees are so inefficient in their work, especially that connected with nest-building, that 8/9 of their efforts are wasted. I cannot agree with Wagner (pp. 136–154) on this point. While it is true that bumblebees do not produce such populous colonies as certain other Social Hymenoptera, they are, as the following observation shows, nevertheless very industrious and efficient creatures. On August 5, 1921, I placed about 50 workers belonging to a large colony of *Bremus impatiens* in a glass-covered box and provided them with honey (in a porcelain dish) and five pieces of old bumblebee comb, each consisting of about a dozen empty cocoons. The bees started at once to repair three of the pieces of comb with wax which they took from the other two. In the course of a few hours, nothing remained of these two pieces of comb except a few shreds, and the remaining three were completely renovated and filled with honey. The rapidity with which this work was carried out shows that Wagner's (1907) claim is an exaggeration.

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[1] Wheeler and Taylor (1921) have found that this is also true of social wasps.

XIX. On the Hibernation Instinct of Bumblebees.

Wagner (1907) claims that the psychic qualities of social insects are no higher, and in some cases even lower, than those of solitary insects. As proof for this contention he cites (p. 196) the "fact" that the queens of bumblebees and social wasps overwinter singly, while certain solitary bees, *e.g. Xylocopa*, hibernate in groups. That these assertions of Wagner (pp. 195–196) are incorrect, at least as far as social wasps are concerned, is shown by recent observations of Mr. and Mrs. Rau (1918), who found that the queens of some of our North American wasps congregate for hibernation; and, as the following observations indicate, there also seems to be a tendency in this direction in bumblebees. In artificial overwintering experiments, Lindhard (1912) found as many as eleven *Bremus* queens hibernating close together, and I was able to make similar observations out in the open. On October 15, 1922, I dug up—within an area of less than two square feet—21 queens of *Bremus impatiens*, many of which were hibernating in groups of two and three. In dealing with this question, Wagner (p. 196) furthermore overlooked the fact that bumblebee colonies in certain parts of the tropics are probably perennial (cf. von Ihering, 1903).

Equally incorrect is Wagner's (p. 9) surmise that a spot free from grass constitutes one of the conditions for the young bumblebee queen in the choice of her hibernaculum, for the spot in which the 21 queens were hibernating was completely covered with grass.

XX. The Position of the Nest and Hibernation.

Wagner (p. 7) states that it depends upon the fall weather when bumblebees begin to hibernate, and that queens reared in well-protected nests (*e.g.* those in the ground or in hay stacks) take up their winter quarters later than those reared in surface nests. These two statements are likewise incorrect. In Europe, the queens of *Bremus pratorum*, a species which frequently nests in the ground (cf. Armbruster, 1914, p. 686), begin to hibernate in July (cf. Sladen, 1912, p. 12), while those of such surface-nesting species as *agrorum* and *muscorum* do not begin to hibernate until at least a month later. The same thing holds true for some of our American species. Here in the vicinity of Boston, the nests of *Bremus bimaculatus* and *Bremus terricola* are almost always subterranean, and yet the majority of the queens of these two species take up their winter quarters in July, while those of *Bremus fervidus* and *Bremus vagans*, species which usually nest on the surface of the ground, do not begin to hibernate until the latter part of August. These facts indicate that the beginning of hibernation depends on the species rather than on the weather and the position of the nest.

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THE FUNCTIONS OF THE SWIMBLADDER OF FISHES.

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Introduction.

The swimbladder, or air-bladder, of a fish is situated dorsal to the cœlom, between the alimentary canal and the vertebral column. It is a membranous sac containing the atmospheric gases. On a portion of the surface of the bladder there is a vascular and glandular area, known as the *rete mirabile*, or "gas gland," or "red gland." Embryologically the swimbladder arises as a diverticulum from the dorsal side of the alimentary canal. The primitive connection may be permanently retained as a tubular canal, called the *ductus pneumaticus*, or it may be entirely absent in the adult. Because of this variation the teleostean fishes have been grouped into two divisions—the Physostomi, characterized by the retention of the duct, and the Physoclisti, in which the duct is absent in the adult. In the former group are included the carp, salmon, and eel; in the latter, the cod, bass, and perch. The basis for this distinction between the two groups, however, is far from invariable and many exceptions occur in both.

The variety of functions performed by the swimbladder is perhaps greater than that of any other organ possessed by fishes.

Among the important functions assigned to the swimbladder are: (1) phonation, or sound producing; (2) respiration; (3) accessory audition; (4) hydrostatic activities.

The respiratory function is believed by most scientists to be important only in a few species. The sound-producing function also has been demonstrated in a limited number of species. Apart from the function of phonation, the swimbladder occasionally becomes subservient to the auditory organ, with which it may be connected directly or through the interpolation of certain modified parts of the anterior vertebræ. The most important function of the swimbladder is hydrostatic, by virtue of which a fish possesed of such an organ is able to alter the amount of contained air and consequently its own specific gravity, so as to be in equilibrium with the surrounding medium under varying pressures.

The investigations to be described in this paper began during the summer of 1921 while the author was in the employ of the United States Bureau of Fisheries. From results obtained that summer and from suggestions gained from Dr. A. S. Pearse, it was thought worth while to continue the study of the functions of the swimbladder. Pearse (1920) had previously shown that the swimbladder gases change in percentage, oxygen becoming less, when perch are kept in water containing small amounts of dissolved oxygen. An attempt was first made to ascertain how important this fact might be in explaining how fishes are able to go into regions where the oxygen is extremely low or entirely absent and live for several hours. The problem developed from that work and was finally extended to cover other functions which the swimbladder might perform. The experiments were restricted to yellow perch, *Perca flavescens* (Mitchell); large mouth black bass, *Micropterus salmoides* (Lacépéde); and carp, *Cyprinus carpio* (Linnæus); A few comparisons were made with other species inhabiting Lake Mendota.

HISTORICAL.

The rôle that the swimbladder plays in the life of fishes has attracted the attention of investigators for a long time. Aristotle believed the organ to function chiefly in the production of sounds. Borelli (1680) was perhaps the first to attribute to the swimbladder an hydrostatic function. He maintained that the fish possessed a volitional control over the size of the swimbladder—being able to compress or distend the bladder at will. Delaroche (1807) opposed the views of Borelli but advanced an hypothesis similar to it in many respects. Moreau (1877), however, maintained that he had disproved both these hypothesis. Delaroche was among the first to show that an exchange of gas probably occurred between the swimbladder and the blood. He did not consider the former an organ of respiration however.

Biot (1807), Provencal and Humboldt (1809) showed by analysis of the air in the swimbladder that the mixture frequently consisted almost entirely of oxygen, the percentage of oxygen increasing in relation to the depth of the water in which fishes lived. Moreau (1877) in his classical work of the function of the swimbladder proved by ingenious experiments that many of the ideas prevailing before his time were erroneous. He showed that this organ serves to equilibrate the body of the fish with the surrounding water at any level. He demonstrated that such adjustment is not accomplished quickly, and that the fish, therefore, does not use his muscles in regulating the volume of the swimbladder. Moreau's experiments also convinced him that the gas is secreted into the swimbladder. About the same time Johannes Müller (1842) took up the problem. Although many of his views have later been shown untenable, they served as an incentive to other investigators. He stated that the gas entered the swimbladder by secretion from the blood.

Several views have been held concerning the origin of gases in the swimbladder. The different theories can be classified into two distinct categories: (1) Those that hold that the gases are derived directly from the atmosphere, attributed first to Redi (1684); (2) and those that maintain that these gases are derived more or less directly from the blood stream (a descendant of Needham's secretory theory).

The first view has been given up by most modern investigators, although Thilo (1906) has persistently contended that the blood of fish is not sufficient in quantity to supply the amount of gas found in the bladder. He asserts that in all cases the bladder-gas must be procured by the fish directly from the atmosphere. Objections are easily found to Thilo's hypothesis.

For example the percentage composition of the three gases, nitrogen, oxygen, and carbon dioxide, is quite different in the swimbladder from that present in the atmosphere. Thilo does not explain how the degeneration of the ductus pneumaticus in the Physoclisti can be correllated with their ability to change their specific gravity.

The second class of theories contains three distinct views as to how the bladder-gases are derived from the blood stream: (1) those which suppose that the blood gives up its gaseous constituents more or less directly to the bladder, the gas passing directly from the blood into the bladder lumen; (2) those that suppose that the gas gland is a pumping organ that is able to force gases from the blood into the swimbladder gland; and (3) those that hold that the gases of the swimbladder are derived from the cytoplasmic decomposition of the gas gland cells.

The first view which is often associated with the name of Moreau (1877), is now quite out of date, although references are often made to it in textbooks and other works of general nature. The second and third views are still current and have excited much controversy. Both of these suppose that the gas gland is the special organ which extracts the gas from the blood. They have been held for several years with great tenacity by schools represented by Jaeger, and opposed by the adherents of Nusbaum and Reis.

Jaeger (1904, 1908) holds that the gas gland is primarily a pumping apparatus, an apparatus for pumping the gases contained in the blood into the swimbladder cavity. He further supposes that the disintegration of a certain percentage of red blood corpuscles is effected by secretions from the gas gland cells —a toxin being poured into the blood for this purpose.

Nusbaum (1907) and Reis (1906) as a result of extensive studies of the cytology of the gas gland have formulated certain peculiar views as to its functions. They contend that gas bubbles are produced by actual decomposition of the substance of the gas gland cells, just as gases are produced by putrefaction. They also believe that erythrocytes are taken into the cells of the gas gland and decomposed, oxygen being liberated and used for such decomposition. There are many objections to such a theory. For example, nitrogen is not known to be formed by decomposition and yet a large percentage of the swimbladder air is usually nitrogen.

Woodland (1911) attempted to compromise the Jaeger-Nusbaum controversy. He assumed that a lysin was secreted by the gas gland cells. This he supposed to hemolyse the erythrocytes and thus liberate oxygen which the gas gland cells could hold and thus force into the swimbladder lumen. Woodland refuted his own hypothesis in the following year however' (1912) and concluded "that hemolysis does not occur in the gas gland," but he offered no suggestion as to how gas does enter the swimbladder. It is obvious that a more or less continual hemolysis or a disintegration of the erythrocytes would be a severe drain on the blood, and it seems probable that some other explanation, more economical for the organism, could be found. The writer attempts to furnish such an explanation in the present paper.

General Methods.

In the study of the functions of the swimbladder of fishes reported in the following pages the attempt was made to employ standard biochemical and physiological methods. In many cases, however, special apparatus was needed. Such apparatus was devised and is described in connection with the respective experiments.

In the analyses of the composition of the gases within the swimbladder, the Haldane (1912) gas analysis apparatus was used; the Newcomer (1919) modification being found to be the most convenient form. A burette holding five cubic centimeters was used instead of the one with the usual ten cubic centimeter capacity, for the reason that the quantity of gas obtainable from the swimbladder is, in most of the fishes used, less than ten cubic centimeters. The sample was drawn from the fish into a five cubic centimeter sampling tube (Fig. 1.) The sampling tube *S* connected with a levelling tube *L* and a hypodermic needle *H* were filled with mercury. The hypodermic needle was thrust through the body wall directly into the swimbladder. The stopcocks were opened, the levelling tube lowered, and the gas thus drawn into the sampling tube. The stopcocks were then closed. The samples were introduced into the gas analysis apparatus the same day and analyzed in the usual manner.

The methods for determining gases in water were similar to those described by Birge and Juday (1911). Many of the experiments were conducted in the field in order to study the fishes in their natural habitats. Samples were taken from a boat and although at first this seemed somewhat inconvenient, it was later found that little difficulty accompanied the operations. Temperatures, soundings, etc., were recorded at the time the experiments were performed.

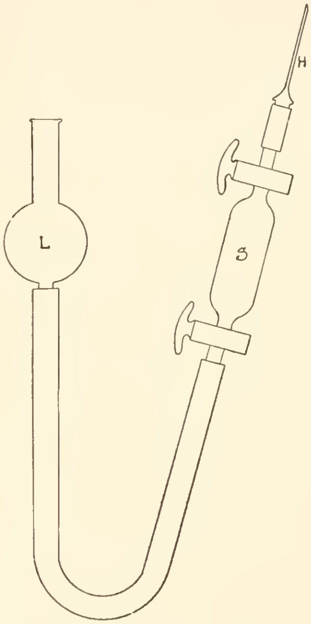


Fig. 1. Swimbladder gas collecting tube.

The Normal Composition of Gases in the Swimbladder.

It was of course important to know the normal composition of the gases in the swimbladders of the species under investigation. The gases vary with the species, with the dissolved gases in the surrounding media, with varying temperatures and pressures, and apparently with other factors that are not yet understood. Probably, also, the degree of salinity of the water, the season of the year, and the physiological condition of individual fishes (influenced directly or indirectly by factors already mentioned) act to produce variations in the composition of the gases found normally in the swimbladder of any particular species.

An attempt was made to study the normal composition of the gases of the swimbladder of several species of fishes under as nearly natural conditions as could be obtained. Fishes were caught in gill nests and traps and were immediately conveyed and transferred to a wire cage sunk near the surface of the water in Lake Mendota. The location of the cage was previously tested for its suitability. The fishes were left in this cage from 48 to 72 hours. Samples of air content of the swimbladder were then taken by methods already described. The amounts of dissolved gases, temperature, and depth, were recorded at the time the samples were taken. The results are summarized in Tables I. and II.

TABLE I.

NORMAL PERCENTAGES OF THE GASES OF THE SWIMBLADDER OF PERCH AFTER BEING KEPT IN A WIRE CAGE FOR 72 HOURS IN LAKE MENDOTA.
SEPTEMBER, 1921.

No.	Date.	Gases in Water.		Depth, Meters.	Temp. ° C.	Gases in Swimbladder.	
		O_2.	CO_2.			Per Cent. O_2.	Per Cent. CO_2.
20	1	4.8	−2.53	.6	24.8	17.4	0.60
21	1	"	"	"	"	21.5	0.66
22	1	"	"	"	"	15.6	0.46
23	1	"	"	"	"	16.8	0.60
32	4	4.8	−2.53	.6	24.8	12.9	1.15
33	4	"	"	"	"	25.1	0.77
34	4	"	"	"	"	21.8	1.03
35	4	"	"	"	"	22.3	0.96
36	4	"	"	"	"	9.5	0.44
37	4	"	"	"	"	23.0	0.62
38	5	4.6	−1.01	.6	22.0	28.0	0.86
39	5	"	"	"	"	22.6	0.69
40	5	"	"	"	"	17.4	0.53
41	5	"	"	"	"	19.8	0.65
42	5	"	"	"	"	22.0	0.68
43	5	"	"	"	"	21.2	0.66
44	5	"	"	"	"	22.0	0.62
45	6	5.8	+2.20	.6	23.0	18.8	0.63
46	6	"	"	"	"	24.0	0.58
47	7	"	"	"	"	25.4	0.45
48	7	"	"	"	"	17.2	0.60
49	7	"	"	"	"	15.2	0.66
50	7	"	"	"	"	20.8	0.39
51	7	"	"	"	"	25.2	0.62
52	7	"	"	"	"	23.5	0.43
53	7	"	"	"	"	24.8	0.43
54	7	"	"	"	"	22.6	0.64
55	7	"	"	"	"	10.3	0.34
Averages		5.1	0.30	.6	23.4	19.9	0.63

TABLE II.

THE RESULTS OF ANALYSES OF THE SWIMBLADDER GASES OF VARIOUS SPECIES OF FISHES SEINED IN UNIVERSITY BAY, LAKE MENDOTA, NEAR THE SURFACE, SEPTEMBER 12–14, 1921.

The dissolved gases ranged as follows: Oxygen = 3.8–4.0 c.c. per liter; Carbon dioxide = 1.7–2.0 c.c. per liter. The temperature was 22.2° C.

Species.	No.	Gases in Swimbladder.	
		O_2.	CO_2.
Pickerel	1	42.9	2.10
Blue-gill	1	14.8	0.48
Sunfish	1	17.6	0.60
	2	24.2	0.56
	3	22.1	0.28
	4	21.2	0.81
	5	22.2	0.49
	6	24.3	0.71
	7	21.9	0.68
	8	20.9	0.67
	9	23.5	1.07
Largemouth black bass	1	19.2	0.52
	2	22.4	1.00
	3	25.7	0.86
Rock bass	1	20.4	0.86
	2	21.5	1.06
	3	21.2	0.31
	4	21.3	0.97
	5	20.4	0.64

At different times during the year, fishes were taken from tanks in the laboratory and the gas content of their swimbladder analyzed for the purpose of controls for various experiments. The tanks were supplied with water pumped from Lake Mendota. The water was well aerated before it was introduced into the tanks, because that coming directly from the pipes was supersaturated with atmospheric gases. The fishes were kept for long periods of time in these tanks in apparently good condition, although for most of the experiments fishes were used that had been brought in from the lake within a period of from 10 to 14 days. The results of the analyses of apparently normal fishes taken from laboratory tanks are summarized in Table III.

It is shown that the normal compositions of gases in the swimbladder of perch was 19.9 per cent. oxygen and 0.63 per cent. carbon dioxide when the fishes were kept in a normal environment.

Table III.

The Results of Analyses of the Swimbladder Gases of Various Fishes Kept in Laboratory Tanks.

The temperature varied from 20 to 23 degrees centigrade. August and September.

Species.	Dissolved Gases c.c./liter.		Gases in Swimbladder.	
	O_2.	CO_2.	Per Cent. O_2.	Per Cent. CO_2.
Small mouth Black Bass . . .	5.5	N	18.9	22.3
Sucker	6.3	N	5.4	4.35
Carp	4.93	4.5	4.2	2.5
	4.93	4.5	3.8	3.1
	4.93	4.5	2.6	3.7
	4.93	4.5	3.2	2.8
	5.5	N	7.3	5.4
	5.5	N	12.7	5.7
	5.5	N	7.6	4.5
	5.5	N	7.9	4.3
	4.6	N	5.3	5.8
	5.4	N	4.9	3.0
	5.3	N	1.8	0.8
	3.9	.76	7.4	3.3

The Effects of Oxygen Deficiency.

Pearse (1920) showed that perch, even though they are able to recognize the proportion of oxygen and carbon dioxide in water, enter regions where conditions are unfavorable for respiration and may remain there in oxygen-free water for as much as two hours without dying. He also showed that the oxygen supply of the swimbladder was depleted when the fishes were subjected to waters of low oxygen content. On his suggestion further investigations were carried on during the summer of 1921.

A search was made, in Lake Mendota, for a region of low oxygen. Such an one was found at the mouth of University Creek, in University Bay. This creek empties into the southern end of the bay in shallow water behind a bar. During the summer months a thick covering of duckweed (*Lemna* and *Wolffia*) may be found there. The position of this plant covering is governed by the direction of the wind, but whenever a wind from the North, Northwest, or Northeast is blowing, or when there is little or no wind, one may prophecy with a fair degree of assurance that the mouth of University Creek will be covered with a mat of this duckweed which prevents the processes of photosynthesis from going on below. Thus a region of very low oxygen is produced.

A wire cage, three feet high, three feet wide, and three feet in length, was sunk in this region. The experimental fishes were placed in this cage, having been previously kept in a cage of similar dimensions in regions of more normal oxygen content. The fish were left in the cage from one to two hours as recorded in Table IV., which shows the results of the experiments.

Another series of experiments was carried on in the laboratory. A large still of thirty-five gallons capacity, loaned by the Wisconsin State Prohibition Commissioner, was used for obtaining oxygen-free water. The water was brought to the boiling point and siphoned through a series of condensers. The water was conveyed directly from the condensers to an aquarium which was kept at the same temperature as the water in which the fish were accustomed to live. Fishes were placed in this aquarium and removed at intervals. The gaseous content of the swimbladder and the amount of dissolved gases in the water were determined from samples taken simultaneously. The results of these experiments are shown in Table V. Control fishes were kept in aquaria at the same temperature and where other conditions were as similar as possible to that in the aquaria where fishes were subjected to low oxygen. The swimbladder analyses of ten controls averaged 16.0 per cent. oxygen and 0.74 per cent. carbon dioxide.

The results of these experiments show that when perch were subjected to water of low oxygen content in Lake Mendota averaging 2.1 cubic centimeters per liter, the percentage of oxygen decreased, and was 12.1 per cent, as compared with the normal of 19.9 per cent. When perch were subjected to water containing extremely small amounts of oxygen, averaging 0.67 cubic centimeters per liter, in aquaria in the laboratory, the percentage of oxygen in the swimbladder decreased and was 9.78 per cent. as compared with normal controls having 16.0 per cent. oxygen.

These results lead one to speculate concerning to what extent the ability of the fishes to re-absorb oxygen from the swimbladder explains how such fishes are able to go into regions of low oxygen content and live for several hours. The question can be answered tentatively by certain theoretical considerations. A normal perch of average size has a swimbladder capacity of

TABLE IV.

ANALYSES OF THE GASES OF THE SWIMBLADDERS OF PERCH SUBJECTED TO WATERS OF LOW OXYGEN CONTENT, IN A WIRE CAGE IN LAKE MENDOTA.
SEPTEMBER 7 TO 15, 1921.

No.	Gases in Water.		Depth, Meters.	Temp., ° C.	Gases in Swimbladder.	
	O_2.	CO_2.			Per Cent. O_2.	Per Cent. CO_2.
56	0.85	+15.4	0.7	22.0	17.4	1.37
57	"	"	"	"	17.2	1.63
58	"	"	"	"	16.5	1.20
59	"	"	"	"	11.0	1.17
60	"	"	"	"	15.4	1.36
61	"	"	"	"	18.2	2.45
62	"	"	"	"	19.0	2.04
63	1.9	+11.6	"	21.0	14.0	1.79
64	"	"	"	"	9.8	1.57
65	"	"	"	"	16.3	1.25
66	"	"	"	"	12.2	2.93
67	"	"	"	"	7.3	1.00
68	"	"	"	"	11.3	0.89
69	"	"	"	"	10.8	1.03
70	"	"	"	"	9.5	1.37
71	1.45	+10.1	"	21.8	13.8	2.04
72	"	"	"	"	21.3	1.76
73	"	"	"	"	12.8	1.86
74	"	"	"	"	13.8	2.00
75	"	"	"	"	21.7	1.89
76	"	"	"	"	15.7	2.62
92	1.6	+10.1	0.8	19.1	6.3	1.47
93	"	"	"	"	15.5	0.73
94	"	"	"	"	11.1	1.81
95	"	"	"	"	9.0	1.39
96	"	"	"	"	8.0	1.49
97	"	"	"	"	7.9	1.41
98	"	"	"	"	8.8	1.23
99	"	"	"	"	9.2	1.21
100	"	"	"	"	11.9	2.03
109	4.1	+12.6	"	18.0	14.5	1.48
110	"	"	"	"	8.7	1.05
111	"	"	"	"	8.0	1.37
112	"	"	"	"	7.0	1.28
113	"	"	"	"	7.6	1.19
114	"	"	"	"	12.7	1.48
115	"	"	"	"	5.0	1.24
116	"	"	"	"	7.5	1.43
117	"	"	"	"	8.6	1.44
Averages	2.1	+11.9	0.74	20.2	12.1	1.54

Table V.

Analyses of the Gases from the Swimbladders of Perch Subjected to Water of Low Oxgyen Content.

Experiments were performed in a laboratory aquarium. Water made oxygen-free by boiling and fishes were left in it for varying periods of time. *N* indicates normality, *i.e.*, no free carbon dioxide.

No.	Date, 1922.	Dissolved Gasses in Water.		Temp., ° C.	Time, Hrs.	Gases in Swimbladder.	
		O_2.	CO_2.			Per Cent. O_2.	Per Cent. CO_2.
1	March 20	0.7	*N*	7.0	5	9.8	1.40
2	“ 20	0.7	*N*	7.0	5	6.9	1.34
3	“ 26	1.0	*N*	6.0	6	12.2	2.08
4	“ 26	1.0	*N*	6.0	10	16.8	2.30
5	April 1	1.1	*N*	6.0	5	13.3	2.40
6	“ 1	1.1	*N*	6.0	8	18.8	5.40
7	“ 2	0.8	*N*	6.0	6	5.4	2.50
8	“ 2	0.8	*N*	6.0	9	15.4	2.40
9	“ 12	0.6	*N*	7.0	5	9.4	0.22
10	“ 13	0.7	*N*	7.0	6	7.8	0.62
11	“ 16	0.7	*N*	7.0	3	16.4	0.92
12	“ 16	0.7	*N*	7.0	3	8.0	0.31
13	“ 16	0.7	*N*	7.0	4	14.9	0.25
14	“ 16	0.7	*N*	7.0	4	5.4	0.12
15	“ 17	0.3	*N*	7.0	4	11.2	0.38
16	“ 17	0.3	*N*	7.0	4	2.85	0.12
17	“ 17	0.3	*N*	7.0	4	5.9	0.55
18	“ 18	0.3	*N*	7.0	4	2.8	0.14
19	“ 18	0.3	*N*	7.0	6	1.72	0.78
Averages....		0.67	*N*	6.7	5.3	9.73	1.27

approximately ten cubic centimeters. The average percentage of oxygen was found to be 19.9 per cent. In other words the actual amount of oxygen would be about two cubic centimeters. From work on the rate of respiratory exchange in perch (to be reported in a later paper) the oxygen consumption was found to be 61.8 cubic centimeters per kilogram of weight per hour at 3° C. The average perch weighs somewhat less than 100 grams. Thus the amount of oxygen required would be about six cubic centimeters of oxygen per hour. The amount that the swimbladder could furnish is only a fraction of that amount. We must conclude, therefore, that although the perch may draw on the swimbladder for oxygen when in regions of low oxygen, the amount that can be furnished to the blood by re-absorption is not enough to keep the fish alive for more than a fraction of an hour. Some other explanation must be found for the reason why fishes are able to survive for longer periods of time in regions of low oxygen content.

Effects of Pressure.

That pressure would affect the percentage of gases in the swimbladder of fishes has been long known. Biot (1807), Provencal and Humboldt (1809) found that the amount of oxygen in the swimbladder varied from 1 to 87 per cent., the percentage increasing with the depth. The classical work of Moreau (1842) showed that the fish accommodate themselves to changes in pressure very gradually, and can live comfortably at varying depths.

Only a few experiments are reported here to show that the response of perch to pressure results in the same increase in oxygen as reported by other investigators for other fishes. It was observed that fishes caught in gill nets at a depth of nine meters in Lake Mendota showed a much higher oxygen percentage than those caught near the surface. The averages of nineteen perch caught at nine meters, during month of August, 1921, were:

Oxygen = 34.7 per cent., Carbon dioxide = 0.60 per cent.,
Nitrogen = 64.7 per cent.

The averages of twenty-eight perch kept at the surface in Lake Mendota were:

Oxygen = 19.9 per cent., Carbon dioxide = 0.63 per cent.
Nitrogen = 79.5 per cent.

From the data of Birge and Juday (1911) the amount of dissolved oxygen at this season of the year was less at nine meters depth than at the surface. Thus it appears that the cause of this increase in the amount of oxygen in the swimbladder can be attributed to pressure.

An apparatus was constructed in the laboratory to study the effects of pressure as a factor in producing increase in the percentage of oxygen in the swimbladder, other conditions being equal. A steam pipe, eight inches in diameter and thirty-six inches long, was closed at one end and fitted with a cap at the other end. In this cap was fitted a glass window four inches in diameter through which the fish could be observed. Pipes running from the fourth floor of the Biology Building were connected with this tank, one carrying water to the tank, one carrying water away from the tank. The height of the overflow pipe was approximately sixty feet above the tank. Thus a pressure was obtained in the tank equal to that at a depth of sixty feet in the lake, or at about two atmospheres greater than at the surface. The fish could be very quickly removed and samples taken for analysis.

Six controls that were kept in water containing the same amounts of dissolved gases and at the same temperature as the fishes subjected to pressure showed the following averages for gases in their swimbladders:

Oxygen = 12.1 per cent., Carbon dioxide = 0.65 per cent.,
Nitrogen = 87.5 per cent.

The averages for seven perch under sixty feet of water pressure at the end of ten hours duration were:

Oxygen = 18.5 per cent., Carbon dioxide = 0.50 per cent.,
Nitrogen = 81.0 per cent.

This shows that with an increased pressure the percentage of oxygen is increased.

THE EFFECTS OF INCREASED CARBON DIOXIDE.

Carbon dioxide is found in very small amounts in the atmosphere (0.08 per cent.). It is found more abundantly in water on account of its ready solubility. The amount in the water, however, is governed by the partial pressure of the gas in the atmosphere. The free carbon dioxide dissolved in natural waters is affected by photosynthesis and by organic decomposition.

Carbon dioxide is also found in natural waters in chemical union. It is found in two states (Birge and Juday, 1911): that united with a base in the form of normal- or mono-carbonate such as $CaCO_3$ or $MgCO_3$ and is known as "fixed" or "combined" carbon dioxide; and that which converts the monocarbonate into a bicarbonate, called "half-bound" or "bicarbonate" carbon dioxide. The half-bound is of course not in such stable union as the fixed. Although plants can make use of the bicarbonate for photosynthesis it can be of very little importance to fishes. It is only the free carbon dioxide that is considered in the following experiments.

The determination of carbon dioxide is not especially accurate when done by titration methods and can not always be relied upon for absolute results. Probably the most accurate method is that of Pettenkofer's described and modified by Birge and Juday (1911). Such a method was used in these experiments.

Carbon dioxide has a marked effect upon fishes. As in other animals the physiological effect is that of a narcotic, stimulating in small quantities and intoxicating in larger quantities. Carbon dioxide and acids apparently produce similar effects when judged by the behavior of fishes. Reuss (1910) working with varying amounts of carbon dioxide upon rainbow trout found that the effects were similar to those upon birds and mammals. Shelford (1918) also showed that when individuals of several species where dropped into water containing 168 cubic centimeters of carbon dioxide per liter, the first effect was one of stimulation but in a few minutes fishes lost their power to perform correlated movements and died. Shelford also showed that fish display a preference for low carbon dioxide and that they react very definitely to amounts naturally found in ponds and lakes. He concludes that the carbon dioxide content of the water is the best single index of the suitability of the water for supporting fishes.

A study was made of the effects of carbon dioxide in the surrounding water on the gases in the swimbladders of perch. The experiments were carried on in the laboratory. Carbon dioxide gas was bubbled through a large carboy of water. A constant flow of water was kept through the carboy to an aquarium containing the experimental fishes. Individual fishes were removed at intervals and analyses made of the swimbladder gases. Water samples were taken at the same time. All the experiments were begun by having normal lake water in the aquarium and gradually increasing the amount of treated water. The results are shown in Table VI. In every case the oxygen and carbon dioxide in the swimbladder both increased as the carbon dioxide in the surrounding water increased.

Table VI.

Analyses of the Swimbladder Gases of Perch Showing the Effects of an Increased Amount of Carbon Dioxide in the Water.

The perch were placed in an aquarium when the water was at the neutral point. The carbon dioxide was gradually increased and the amount recorded represents the amount dissolved in the water at the time a fish was removed for analysis.

No.	Gases in Water.		Temp., ° C.	Time.	Gases in Swimbladder.	
	O_2.	CO_2.			Per Cent. O_2.	Per Cent. CO_2.
1	10.1	+ 13.7	8.5	3 hrs.	15.6	1.06
2	10.4	+ 21.5	10.0	6 "	12.7	1.72
3	11.7	+ 35.4	10.0	9 "	17.7	1.64
4	12.4	+102.5	9.0	12 "	15.3	7.15
5	11.1	+126.5	9.0	14 "	16.4	10.40
6	11.9	+ 55.7	14.0	2 "	15.8	4.08
7	16.5	+ 72.3	12.0	4 "	14.8	5.80
8	16.5	+ 93.5	11.5	6 "	15.6	6.60
9	16.6	+117.0	11.0	8 "	14.8	8.05
10	17.5	+110.0	11.0	10 "	21.1	5.90
11	7.2	+ 43.0	12.0	3 "	16.1	2.25
12	7.2	+ 60.0	12.0	4 "	17.9	4.30
13	7.6	+ 83.5	12.0	5 "	18.1	5.25
14	7.6	+ 83.5	12.0	6 "	19.7	6.0

It was observed during the preceding experiment that fishes would rise in the aquarium as the carbon dioxide was increased, and this suggested that the specific gravity of the fish perhaps changed. It may also be seen that the oxygen percentage remained fairly constant, indicating that the volume was apparently increasing. An apparatus was devised to determine if the volume of the fish did actually increase. A diagram of the apparatus is shown in Fig. 2. A large bottle *C* of three liters capacity was sealed with a rubber stopper *R*; a three-way stopcock *S* and capillary tubing connected the bottle with a tambour *T*. The tambour controlled a marker which recorded on a smoked drum *D* any change in volume that might take place within the bottle. The bottle was immersed in a constant temperature water bath *B*. The bottle was filled with water having the same temperature as that of the constant temperature bath which was of the same temperature as the water in which the fish was accustomed to live. This water contained a normal amount of oxygen. The apparatus was tested several times to make sure that no leak occurred and that no change in volume took place when fishes and normal water were in the bottle or when water containing large amounts of carbon dioxide and no fish was used. There was no indication of any volume change in any of these tests. Individual perch were placed in the bottle, the water having previously been charged with a small amount of carbon dioxide, the rubber stopper pushed tightly into the aperture; the water rose and passed out through the tube *O*, the stopcock was turned so as to connect the bottle with the tambour. The drum was started and a record of the volume change made.

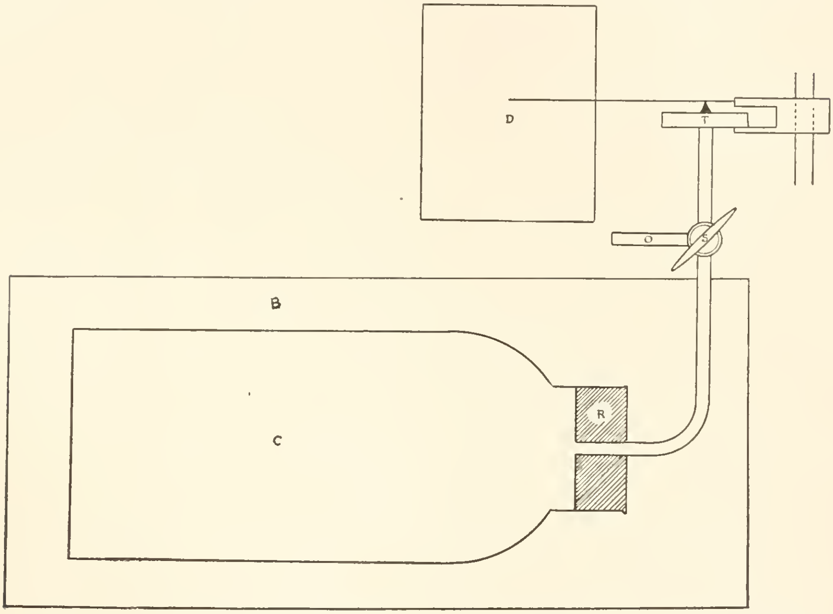


FIG. 2. Apparatus for determining the volume change in fishes. *C*, large mouth bottle; *R*, rubber stopper; *S*, three way stopcock; *T*, tambour; *D*, drum; *B*, constant temperature water bath.

A typical graph is shown in Fig. 4. It will be observed that the most rapid changes took place within the first few minutes. Is this response of the fish to carbon dioxide due to the acid nature of the dissolved gas or to some other property? It is evidently not due to the acid property, for when lactic acid is used instead of carbon dioxide the volume of the fish does not increase, but on the contrary shows a decrease. Fig. 6 shows a graph obtained when lactic acid was added to the water (1:1000). The effects produced by the lactic acid is probably one of constriction of tissues caused by the acid; the effect of carbon dioxide is probably due to diffusion of the gas into the swimbladder.

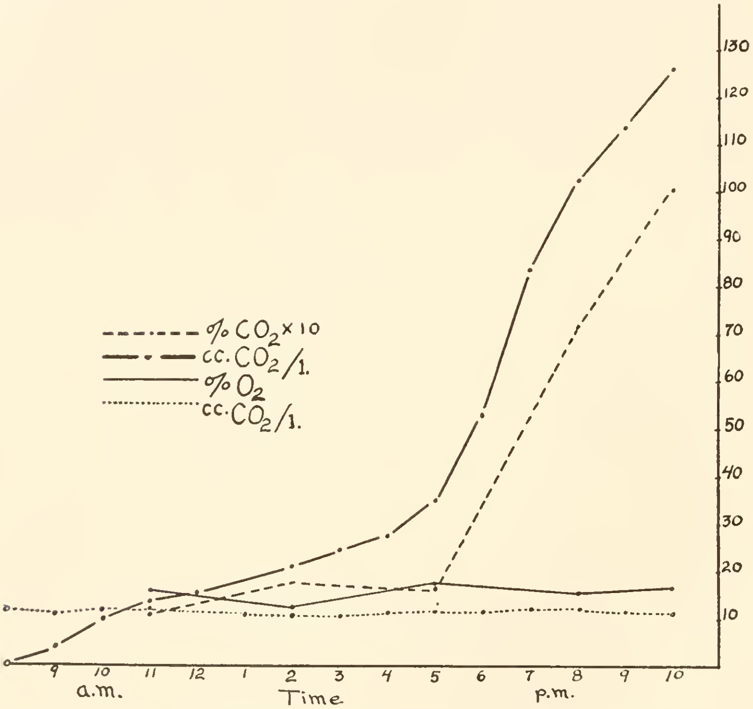


FIG. 3. A graph to show comparisons of the dissolved gases in the water with the gases in the swimbladder with variation of the dissolved gases. The swimbladder gases are expressed in percentages and the dissolved gases in cubic centimeters per liter. A single experiment is represented in the graph. Perch were placed in an aquarium containing normal lake water and the carbon dioxide gradually increased. The time intervals are expressed on the abscissa and the amounts of the gases expressed on the linear scale of the ordinate.

The fact that fishes change their volume when subjected to water containing high amounts of carbon dioxide may be of ecological importance. In many lakes the amount of carbon dioxide dissolved in the water increases with depth. The oxygen decreases with depth and near the bottom may be entirely lacking. Both these factors make the bottom of a lake less favorable for respiration than shallower regions. Of course a high amount of carbon dioxide is toxic and the lack of oxygen may lead to asphyxiation. In Fig. 6, the average carbon dioxide gradient of Lake Mendota is plotted for the month of August from 1906 to 1921. The data were obtained from records kept by President Birge and Professor Juday. It can be seen that the carbon dioxide gradually increases with depth and averages about five cubic centimeters per liter at the bottom. At times it may be two or three times as great as this average figure for Lake Mendota. Such an amount of carbon dioxide may be sufficient to produce an increased volume in the fish and cause it to rise above regions that might be unfavorable for respiration. Shelford (1913) has shown that dissolved carbon dioxide may be taken as an index for the distribution of fishes. He attributes distribution to the results of behavior-regions

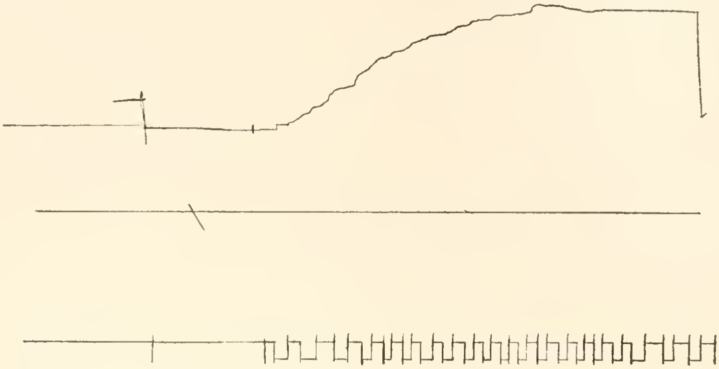


FIG. 4. A graph showing the volume change of a perch when placed in water where dissolved free carbon dioxide was increasing. Time is recorded at two-minute intervals.

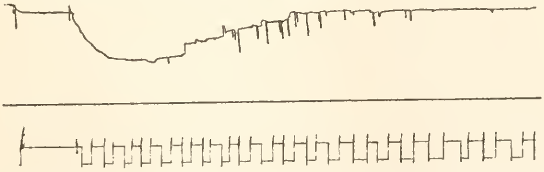


FIG. 5. A graph showing the temporary decrease in volume when a perch was placed in water containing lactic acid (1 : 1000). Time is recorded at two-minute intervals.

high in carbon dioxide being avoided by the fishes. From the experiments described in the present paper, it may be assumed that such distribution is due, at least in part, to a more or less automatic mechanism which operates so as to control vertical distribution by altering specific gravity. Both these processes are ordinarily adaptive and may operate simultaneously.

The results of these experiments show that when fishes are subjected to increased amounts of dissolved carbon dioxide in water, the carbon dioxide in the swimbladder increases and is roughly proportional to that in the water. Fig. 4 illustrates this point. Carbon dioxide produces an alteration in the specific gravity of the fish, causing it to rise and escape from regions which might be unfavorable for its existence.

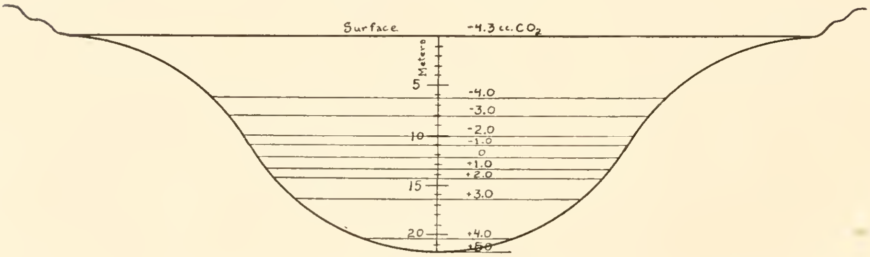


FIG. 6. A diagram to show the carbon dioxide gradients with depth in Lake Mendota for the month of August from 1906–1920. The data were obtained from Birge and Juday. The minus sign indicates alkaline water and the plus sign acid.

DIFFUSION AND SECRETION.

A fluid gives off gas to or takes gas from any other medium with which it is in contact according to the relative pressure of the gases. Dalton's *Law of Partial Pressures* may be stated as follows: in a mixture of gases each gas exerts the same pressure as it would exert if it were alone present in the volume occupied by the mixture. The pressure of each gas is called its partial pressure.

It is believed by most physiologists that the inter-change of gases during respiration is explained by the laws of physical diffusion. The interchange of gases in the lungs has raised a problem that has inspired considerable study. While many physiologists have accepted the view that during the interchange of gases in the lungs, the membranes between the blood and the alveolar air simply play a passive part, and physical diffusion explains the respiratory exchange. However, this view has not gone unchallenged.

Haldane and Smith (1896) claims that the tension of the oxygen in the arterial blood may be higher than the pressure of oxygen in the alveoli. According to the physical theory of respiration, if a permeable membrane separates two volumes of any gas, or two solutions of any gas at different pressures, the molecules of the gas will pass through the membrane in both directions until the pressure is equal on both sides; *i.e.*, gas diffuses from a point of high rension to one of low tension. Haldane maintains that a secretory activity is associated with the function of the lungs, which produces a higher tension of oxygen in the arterial blood than in the alveolar air.

The analogy has many times been drawn between the gas interchange in the lungs and in the swimbladder of the fish. Bohr (1894) punctured the swimbladder and removed gas. He found that if the fish was then left in the water, the gas was rapidly replaced, and, when he tapped the swimbladder a second time, that the percentage of oxygen had greatly increased. Oxygen may amount to between 60 and 80 per cent of the total gas after such an experiment. Bohr found that this reaccumulation did not take place if both vagi were cut and he ascribed it therefore to direct secretory activity on the part of the swimbladder. Bohr is inclined to endow the vagus nerves of the higher vertebrates, including mammals, with an analagous regulatory influence on the gaseous exchange in the lungs.

Starling (1920) strongly maintains that the lungs cannot be considered analagous to the swimbladder. He says there is no likeness between the thick secreting cells of the "red gland" which is apparently the gas-secreting part of the swimbladder, and the thin structureless plates which separate the capillaries of the lungs from the alveolar air.

It was considered necessary to repeat some of Bohr's work during the present experiments, using fresh-water fishes. Yellow perch were used for the first series. Gas samples were obtained by methods already described (p. 000). After the first sample was taken the fishes were placed in the same tank in which they had been accustomed to live. No changes in the amounts of dissolved gases were detected in the water during the experiments A second gas sample was taken from the swimbladder after a varying interval of time. A comparative record of the results are shown in Table VII.

The large black bass and the carp were also used and the results are summarized in Table VII.

TABLE VII.

RESULTS OF EXPERIMENTS WITH THE SECOND REMOVAL OF GASES FROM THE SWIMBLADDER; THE DISSOLVED GASES IN THE SURROUNDING MEDIUM WERE KEPT CONSTANT.

Species.	No.	Gases in Swimbladder, First Removal.		Duration of Time between First and Second Removal in Hours.	Gases in Swimbladder, Second Removal.	
		Per Cent. O_2.	Per Cent. CO_2.		Per Cent. O_2.	Per Cent. CO_2.
Smallmouth black bass.	1	18.9	2.22	24	36.2	4.02
	2	36.2	4.02	24	46.0	3.20
	3	18.7	1.15	48	46.0	5.54
Largemouth black bass	1	20.0	1.48	24	29.7	1.72
	2	18.7	0.72	24	30.0	3.06
	3	17.9	0.88	24	31.3	2.66
Yellow perch.........	1	13.1	0.70	12	16.1	1.57
	2	11.1	0.60	12	35.2	3.10
	3	12.7	0.60	12	23.1	1.70
	4	14.0	0.85	12	25.2	2.00
Carp................	1	4.2	2.5	7	4.8	1.45
	2	3.8	3.1	7	4.8	0.60
	3	8.0	4.3	48	10.7	3.16
	4	5.4	5.1	24	5.7	5.10

The results show that the percentage of oxygen increases in the bass and the perch when second sample is taken after an interval of time. In the carp, however, only slight changes could be observed. The carp has an open duct leading from the swimbladder while the bass amd the perch have closed swimbladders. This is believed to be the explanation for the difference in the response of the two types to withdrawal of air from the swimbladder.

It is apparent that the question of whether oxygen is transferred to the swimbladder by physical diffusion or secretion rests on a knowledge of the partial pressures of the gases in the blood and in the swimbladder. If one can know the partial pressures or tensions of the oxygen in the swimbladder and in the blood coming to the swimbladder he will have the solution of the problem. If the tension of the gases should be the same in the swimbladder and in the blood coming to the swimbladder one could but conclude that the gases were transferred by physical diffusion, but if the tensions were not equal and should be markedly greater on one side than on the other he would be justified in concluding that an active secretion existed.

However, the determination of the gaseous tensions in the blood presents considerable difficulty. It is necessary to bring the blood in contact with gaseous mixtures containing various proportions of the gas whose tension in the blood it is desired to measure. By making various experiments a gaseous mixture will be found with which the blood is in equilibrium. For such determinations an aerotonometer is used. Because fish blood is somewhat difficult to handle and because of the small amounts that are usually obtained the aerotonometer has not been found practicable. Another method has been devised which is very similar in principle to that of the aerotonometer although absolute tensions can not be determined, but rather differences in tensions. However, the results are definite and strictly comparable.

A diagram of the apparatus used is shown in Fig. 7. An equilibration chamber *C*, opening at one end through a single way stopcock, 1, and on the other through a three-way stopcock, *2*, was connected to a levelling bulb, *L*, by rubber pressure tubing. A vertical glass tube, *G*, was inserted in the rubber tube to make visible the column of mercury within. The equilibration chamber could be shifted so that the stopcock, *2*, was down (position A) or so that it was up (position B). *T* represents the position of a test tube during the experiment and *B* a constant temperature water bath surrounding the test tube.

Gas from the swimbladder of the bass which was known to have a high oxygen tension was drawn into the equilibration chamber, *C*, through a hypodermic needle, by filling the entire apparatus with mercury previously and by lowering the levelling bulb, *L*, with the equilibration chamber in position *B* until the mercury column could be seen in the tube *G*.

Blood was drawn from the dorsal aorta of the bass under paraffin oil, care taken that the blood did not at any time come in contact with the air. The blood was oxalated to prevent clotting. The Van Slyke method was used for the determination of the blood gases (Van Slyke and Stadie) (1921). Usually two

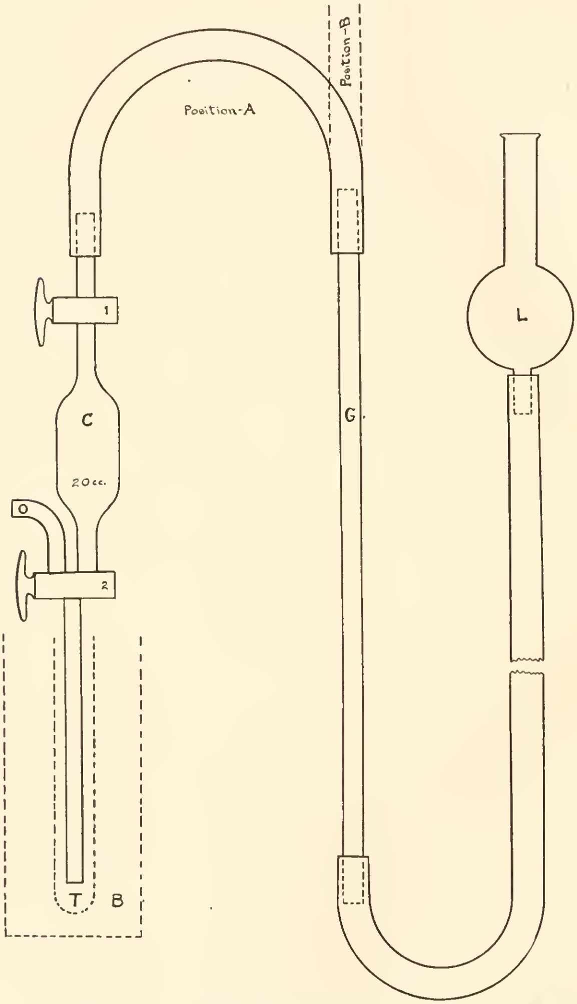


FIG. 7. Blood gas and swimbladder gas equilibration apparatus.

determinations were made to insure accuracy. Following these determinations, the test tube containing the blood was inserted in the position shown in Fig. 7, *T*, (position A). Oil was then drawn up into the tube, through O, to displace the air. The stopcock *2* was then turned to connect the equilibration chamber with the test tube. The levelling bulb was then lowered and the blood in the test tube drawn up into the equilibration chamber *C.* The stopcocks were then closed and the chamber shaken for several minutes until the gases in the blood and the swimbladder gases came into equilibrium with each other. The blood was then allowed to run back into the test tube. Another series of Van Slyke determinations were made. Constant temperatures were maintained throughout the experiments. By this method differences of tensions could be determined. For if the analyses showed an increase or decrease in the determinations before and after equilibration of the blood with the swimbladder they would denote a greater or lesser tension of the gases. The results of the determinations are shown in Table VIII.

TABLE VIII.

RESULTS OF EQUILIBRATION OF FISHES' BLOOD WITH SWIMBLADDER GAS.

Air was previously removed from the swimbladder in order to stimulate secretion. The gas composition recorded represents the percentages at the time the blood was removed from the fishes.

No.	Gases in Swimbladder.		Normal Blood, Vol. per cent.		Blood after Equilibration, Vol. per cent.		Difference, Vol. per cent.	
	Per Cent. O_2.	Per Cent. CO_2.	O_2.	CO_2.	O_2.	CO_2.	O_2.	CO_2.
Bass								
1	46.0	5.54	3.08	20.9	5.74	20.9	+2.66	0
2	30.0	3.06	4.74	22.2	6.47	22.8	+1.73	+0.6
3	32.7	3.40	4.60	21.8	6.41	21.8	+1.81	0
4	41.5	4.30	3.84	22.0	6.16	22.4	+2.32	+0.4
Average	37.5	4.08	4.07	21.7	6.20	22.0	+2.13	+0.25
Carp								
1	4.92	3.00	4.75	8.85	3.07	8.6	−1.68	−0.25
2	7.40	3.32	4.40	12.4	4.82	12.4	+0.42	0
3	1.80	0.75	4.68	9.15	3.02	9.15	−1.66	0
4	5.35	5.80	2.84	10.2	3.05	10.8	+0.21	+0.6
5	6.10	3.87	4.62	11.1	4.88	11.1	+0.26	0
Average	5.11	3.35	4.26	10.34	3.77	10.41	−0.49	+0.07

It will be observed that in experiments with the bass a greater tension of oxygen existed in the swimbladder than in the blood, indicating that an active secretion of oxygen takes place.

In the carp, however, which has a duct from the swimbladder opening into the pharynx, no such tension difference could be detected. Physical diffusion probably accounts for the passage of gases to and from the swimbladder in this fish.

In the normal bass living at the surface, where little or no depth changes are taking place, the percentage of the gases in the swimbladder is probably controlled by simple diffusion. For when determinations were made on such bass the tension of the swimbladder gases and the blood appeared to be the same. It is only when changes such as pressure made a rapid increase of gases in the swimbladder necessary that secretion operated.

Discussion.

The results of this investigation show that in the swimbladder of fishes an active secretion of gases exists, especially of oxygen. There apparently is a regulatory mechanism by which fishes can adjust their specific gravity to that of the environment, thus enabling them to maintain themselves at any particular level with a minimum expenditure of energy. What is this mechanism? Gas secretion has been an extremely controversial subject, especially when reference is made to the lung of the higher vertebrates and the swimbladder has been considered by many to be the homologue of the lung.

It is quite apparent that the gases of the swimbladder are derived from the blood. Oxygen plays the more active rôle in this secretion and nitrogen, which is more inert, is more passive. This at first may seem strange, but if a gas is to be secreted from the blood or absorbed by the blood the advantages are obvious. Oxygen can be easily obtained from oxy-hemolobin and can be used up either by combination with reduced hemoglobin or by oxidation of some reducing substance.

In the mechanism by which the swimbladder derives its oxygen two provisions are necessary: first, the provision for bringing the quantity of oxygen which is required; second, the process by which the oxygen is transferred from the capillary blood vessels to the swimbladder.

The Swimbladder Gland.

If one examines the structure of the swimbladder of certain fishes, at one region or another he will see a glandular structure consisting of a thickened layer of epithelial cells. Below this a more or less conspicuous area, red in color, is visible. This "red body" and its contiguous epithelium is known as the swimbladder gland. To Johannes Müller (1842) may be ascribed the discovery of its true glandular nature. The mass of red blood vessels of the red body, which has for years attracted the attention of anatomists, was discovered by Redi (1684) and described by him. Since his time it has been known by the name of the *rete mirabile.*

Woodland (1911) made a very careful study of the structure of swimbladder glands of many species of fishes. He describes the gland as having two separate parts—the glandular epithelium or gas gland and the *rete mirabile.* The rete may or may not be contiguous with the epithelium. The glandular epithelium may be a single layer or many layered. Woodland found that the arterioles passing to the gland break up into capillaries which come into intimate contact with corresponding venous capillaries from the venules coming from it. There was a free anastomosing of the capillaries on the arterial side with those on the venous side.

The capillaries were regarded for a long time as passive endothelial filters with no muscle and therefore possessing no independent contractility. Krogh (1920) has, however, proved that the capillaries do possess the ability to contract. He has also shown that when many substances are introduced into the blood stream they have marked action on capillaries. Urethane produces extreme dilatation of the capillaries and stasis. Oxygen deficiency leads also to a marked capillary dilatation. Krogh has adduced evidence, from an admirable series of experiments, that the capillaries are normally kept tonically contracted by a substance present in the blood stream. He believes that the pituitary hormone is the substance that maintains the capillary tone, and is present normally in concentration of about one part in a hundred million or less. Krogh also maintains that oxygen, carbon dioxide and probably all crystalloids pass through the capillary walls by purely physical diffusion.

It hardly seems necessary to go further into a discussion of the many experiments that have been performed in this field. It is apparent that when certain metabolic products are thrown into the blood stream because of a heightened metabolism of a gland an increased vascular dilatation ensues, producing a heightened flow of blood to that gland. What application can be made of this process in explaining the mechanism of gas transference in the swimbladder of fishes? Before attempting to answer that question it will perhaps be fitting to mention another means by which the gland is able to regulate the supply of oxygen in proportion to the needs.

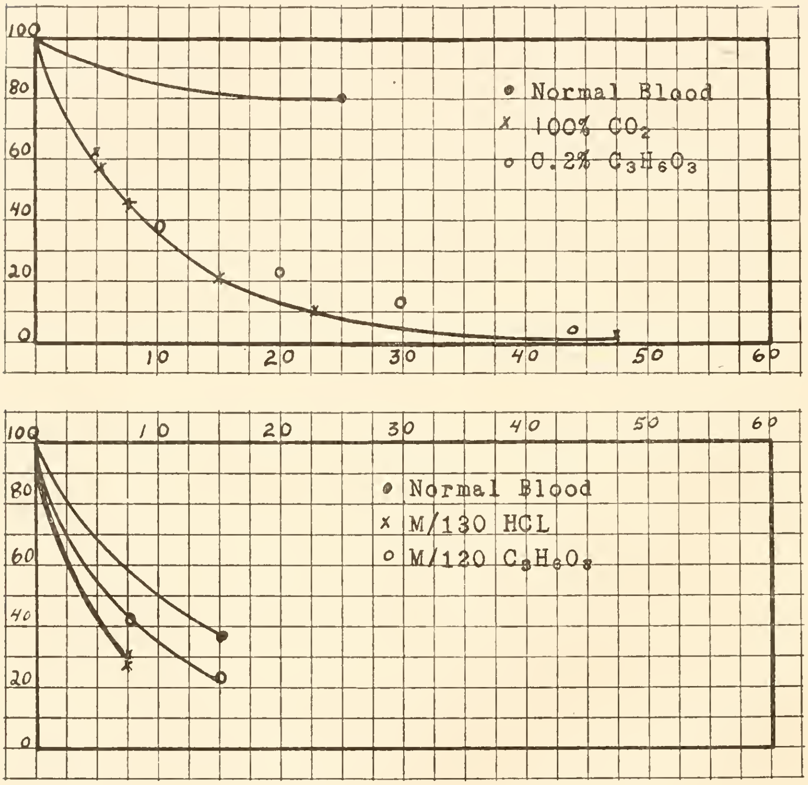
A study was made of the structure of the swimbladder gland of the yellow perch by the writer. It is located on the ventral side of the swimbladder wall near the anterior end. The rete mirabile is seen as a group of small fan-shaped tufts formed by an artery and a vein. A thickened glandular mass of epithelium surrounds the "red body." Micro-photographs (Plate I) also show the mass of capillaries and their terminations in the epithelial cells of the gland.

The blood supply is arterial, coming from either the aorta or the coeliac axis, and in some fishes different portions receive blood from both sources. In the walls of the bladder the arteries break up into the networks of the rete mirabile. From the rete the blood passes to the body veins—postcardinal, hepatic, or vertebral. It seems reasonable to conclude that this remarkable distribution of the blood vessels furnishes an adequate means of supplying the swimbladder with blood for the purpose of secretion.

The second provision—the process by which the oxygen is transferred from the capillary blood vessels to the swimbladder is a more strictly physiological problem. It is evident that the oxygen is first given to the cells of the rete mirabile and then passes out the glandular cells into the swimbladder cavity. This transference of oxygen from the red cell involves a chemical breakdown of the oxy-hemoglobin in the first place, and, following this liberation of oxygen, a diffusion of gas occurs between the blood and the cells. In such a physiological system it is necessary that a definite amount of blood should pass through an organ; it must give a definite oxygen tension to the organ and in so doing becomes reduced. The amount of that reduction varies with the need of any particular organ. The question arises—is there evidence that each or any organ of the body is so far master of its own metabolism that it can force the vascular system to give it the oxygen which it requires. Gaskell (1880) stated this question years ago. Evidence has ever been increasing since his time. Gaskell and other more recent workers have shown that certain metabolic products, especially acids, including carbon dioxide and lactic acid, have the power of distending blood vessels. Dale and his colleagues (1911) have also shown that beta- iminazolylethylamine, a body so closely bound up with the physiology of protoplasm that it is liberated by the splitting off of carbon dioxide from histidine, produces a powerful dilatation of the blood vessels.

Barcroft (1914) and other investigators have shown that the affinity of hemoglobin for oxygen is very sensitive to small changes in acidity or alkalinity. The effect of an increased acidity is to lessen the concentration of the oxygen which is held in solution by the hemoglobin. Barcroft has also shown that the influence of acids depends upon the change in the hydrogen ion concentration which they cause in the blood. Bohr (1896) found experimentally that when the oxygen tension is low, an increase in the carbon dioxide tension tends to dissociate oxy-hemoglobin. Since these conditions prevail in the capillaries, the presence of carbon dioxide in increased amounts facilitates the liberation of oxygen. Barcroft maintains that it is the "reaction" of the blood that has the effect of varying the dissociation of oxygen from oxy-hemoglobin. The more acid the blood contains, or the more acid the "reaction" due to the increased amounts of lactic acid or an increased tension of carbon dioxide, the more readily does the oxy-hemoglobin undergo dissociation.

Two experiments described by Barcroft show that the addition of acids greatly accelerates the reduction of blood and that the concentration of the acids necessary to produce approximately equal effects are those in which they produce equal increments in the hydrogen ion concentration. In Fig. 8 it will be seen that the time taken for mammalian blood containing 0.2 per cent. lactic acid to be reduced by nitrogen, and for blood without lactic acid to be reduced by 100 per cent. carbon dioxide, are indistinguishable. But blood containing either of these solutes requires



FIGS. 8 AND 9. Ratio of reduction of blood with a uniform stream of oxygen free gas. Percentage saturation is shown vertically. Time in minutes is shown horizontally. Fig. 8 shows a comparison with normal blood, with blood containing lactic acid, and with blood reduced by carbonic acid instead of nitrogen. Fig. 9 shows the approximate equality of *M*/130 HCl and *M*/120 lactic acid in their effects. (From Barcroft, 1914.)

about one tenth the time necessary for the same reduction without acids. The blood is reduced from 100 per cent. saturation to 80 per cent. saturation, in about two minutes with the acids present, and about twenty-two minutes with the acids absent.

In Fig. 9 it will be seen that normal blood is reduced (the bubbling was more rapid and the temperature higher) from 100 per cent. to 35 per cent in fifteen minutes, while when it contained $M/130$ HCL and $M/120$ lactic acid the same reduction was effected in about six and one half and seven and one half minutes respectively. Thus it becomes evident that the "reaction," namely the hydrogen ion concentration of the blood effects the reduction of the blood; the amount of dissociation of the oxygen from the oxyhemoglobin is in proportion to the increase in the hydrogen ion concentration.

How may these facts be correlated with the conditions found in the swimbladder of fishes? The abundant supply of blood furnished to the swimbladder by means of the rete mirabile has already been described. The question arises—is the gland which includes the rete mirabile able to force the vascular system to give it the oxygen which it may require in order to maintain a pressure in the swimbladder equal to that at the depth to which a fish is subjected? Does it perform this function by secreting substances causing a dilatation of the blood vessels of the rete mirabile, thus producing an increased flow of blood to the gland, or do these substances accelerate the reduction of the oxy-hemoglobin, or is it a combination of both these processes?

An attempt was made to determine the hydrogen ion concentration changes that might take place in the swimbladder. In making the tests fish were quickly removed from the aquaria and the spinal cord severed just posterior to the brain. The body cavity was opened, the swim bladder gland raised by forceps and cut free from the body, as little time elapsing as possible during the operation. Care was taken not to get any of the body secretions or fluids on the gland. The gland was then placed in a small vial containing about five cubic centimeters of distilled water, which was free from carbon dioxide, which had been previously weighed. The vial was again weighed and the weight of the gland computed. After standing for a definite length of time (see Tables IX. and X.) colorimetric determination of the hydrogen ion concentration was made. The vial was only slightly agitated during the period of standing, in order to keep the gland intact. Three cubic centimeters were pipetted off for the determinations. By this method the changes brought about in the hydrogen ion concentration by substances present in the distilled water were mainly dialyzed from the gland. It is only those substances which do dialyze from the gland that are of concern in this problem. In the first series of experiments the yellow perch was used and a definite amount of gas was removed from the swimbladder (five cubic centimeters) by methods already described in the gas withdrawal experiments, page 83. Care was taken not to injure the fishes more than was absolutely necessary. A very small needle was used and the puncture was made at the opposite end of the swimbladder from the gland.

Table IX.

Determination of the pH of the Swimbladder Gland of Normal Perch.

The gland was allowed to dialyze in 5 c.c. of distilled water free from carbon dioxide, and 3 c.c. was pipetted for the colorimetric determination.

No.	Weight of Gland.	Time of Dialysis.	pH.
1............	0.215 gms.	30 min.	7.1
2............	0.242 "	40 "	6.9
3............	0.156 "	30 "	7.1
4............	0.201 "	30 "	7.1
5............	0.253 "	30 "	7.0
6............	0.194 "	30 "	7.1
Averages..........	0.210 gms.	32 min.	7.05

Table X.

Determinations of the pH of the Swimbladder Gland of Perch following the Withdrawal of 5 c.c. of the Gas from the Swimbladder.

A similar amount of the carbon dioxide free water was used as in the determinations with normal fishes. The "duration of stimulus" of the decreased internal pressure indicates the time between the withdrawal of the gas and the removal of the gland from the fishes.

No.	Duration of Stimulus.	Weight of Gland.	Time of Dialysis.	pH.
1..........	45 min.	9.287 gms.	30 min.	6.4
2..........	2 hrs.	0.285 "	40 "	6.4
3..........	2 "	0.178 "	40 "	6.3
4..........	2 "	0.242 "	30 "	6.4
5..........	2 "	0.257 "	30 "	6.3
6..........	2 "	0.251 "	30 "	6.4
Averages.......		0.250 gms.	33 min.	6.38

Autopsies were made to ascertain the extent of the injury due to the puncturing and in no case was there any apparent evidence of injury to the gland.

As a control in some cases the swimbladders of normal fish were punctured with a sewing needle approximately the size of the hypodermic needle. No gas was removed. The results are shown in Tables IX. and X.

In the second series of experiments fishes were subjected to high pressures for several hours. The apparatus as described previously for pressure experiments, page 91, was used. The pressure in the apparatus registered from thirty-eight to forty-five pounds per square inch. The pressure was applied gradually and removed gradually. The results of these experiments are shown in Tables XI and XII.

TABLE XI.

DETERMINATIONS OF THE pH OF THE SWIMBLADDER GLAND OF NORMAL PERCH.

The gland was allowed to dialyze as in the preceding experiments; the same amounts of the carbon dioxide free water were used.

No.	Weight of Gland.	Time of Dialysis.	pH.
1	0.160 gms.	30 min.	6.9
2	0.535 "	30 "	6.8
3	0.310 "	30 "	7.0
4	0.316 "	20 "	7.0
5	0.305 "	10 "	7.0
6	0.308 "	30 "	7.0
7	0.333 "	30 "	6.9
8	0.314 "	30 "	7.0
Average	0.324 gms.	26 min.	6.96

These determinations show that the hydrogen ion concentration of the swimbladder gland increases when the fish are placed under pressure or when the pressure within the swimbladder is diminished. When five cubic centimeters of the gas was removed from the swimbladder the hydrogen ion concentration of the glands averaged 6.38 as compared with 7.05 of the controls. When fish were subjected to an increased pressure the hydrogen ion concentration of the glands averaged pH 6.64 as compared with pH 6.96 of the controls.

The swimbladder gland consists of connective tissue, a bed of capillaries, and a thick layer of epithelium (Plate I). Increase

TABLE XII.

DETERMINATIONS OF THE pH OF THE SWIMBLADDER GLAND OF PERCH SUBJECTED TO AN INCREASED PRESSURE.

These were made similar to those described in Table XI. The "duration of stimulus" indicates the time to which fishes were subjected to external pressure.

No.	Duration of Stimulus.	Pressure lbs./ sq. in.	Weight of Gland.	Time of Dialysis.	pH.
1.....	40 min.	38	0.122 gms.	30 min.	6.8
2.....	40 "	38	0.205 "	30 "	6.8
3.....	140 "	45	0.194 "	30 "	6.4
4.....	140 "	45	0.210 "	30 "	6.9
5.....	6 hrs.	45	0.392 "	30 "	6.3
6.....	6 "	45	0.405 "	30 "	6.3
7.....	6 "	45	0.745 "	30 "	7.0
8.....	6 "	45	0.330 "	30 "	6.6
9.....	6 "	45	0.365 "	30 "	6.7
Average ..	4 hrs.	43	0.331 gms.	30 min.	6.64

in the acidity is evidently produced in the epithelial cells and acid dialyzes into the capillaries. Two things may be produced in the capillaries: (1) an increase in the calibre of the capillaries; (2) a more complete dissociation of oxygen from oxy-hemoglobin. In either case more oxygen would be furnished to the gland. By increasing the calibre of the blood vessels more blood is brought to the gland and by a more complete oxygen dissociation a greater oxygen tension results. It is not possible to account for the greater oxygen tension in the swimbladder by the first means; by the second, however, where the local tension of oxygen might be so high, it is possible to see how the oxygen tension in the swimbladder might be considerably higher than in the general arterial system. If this hypothesis be correct it is easy to undersrand the purpose of the great mass of capillaries. The function on the thick layer of epithelium is also explained. Just what the nature of the effective acid is can not be stated at present. Perhaps further investigation will reveal its character.

Another observation which seems to support the view just proposed relates to the change in size which the gland undergoes. It is noticeable that there is an increase in the size of the swimbladder glands that have been stimulated. They also appear redder in color. The increase in size is evidenced also by the increase in weight shown in Tables IX.–XII. The average weight for the normal glands of perch was 0.267 grams as compared with 0.290 grams for glands of perch subjected to pressure stimuli. A considerable variation occurs in the cutting of the glands free from the swimbladder, and these figures are not absolute quantitative comparisons; nevertheless they may be taken as indicative.

It is probable that afferent nerves perhaps located in the walls of the swimbladder that are sensitive to changes in pressure aid in the regulation of the specific gravity of the fish. Bohr (1893) showed that the vagus exerts a control over the gas secretion in the swimbladder. Its control is very likely a matter of stimulating the swimbladder gland to secrete. For example when a perch sinks into deeper water the pressure outside increases. In order to maintain its equilibrium, the perch must increase the amount of gas in the swimbladder. It does this by taking oxygen from the blood. When the fish rises in the water oxygen must be re-absorbed by the blood. When the fish is not changing its depth appreciably the gas content of the swimbladder is probably maintained by simple physical diffusion of the gases from the blood, but when a greater difference of pressure is met with the secretory process comes into play.

The mechanism by which those fishes which have a duct leading from the swimbladder maintain their equilibrium in the water has not been studied in detail. Whether the process of gas diffusion is fundamentally different in such fishes can not be stated. Further investigation will be made using such fish as carp, dogfish, and trout. In all events some modifications may be expected for no such highly specialized organ as the rete mirabile exists in these forms.

The writer's hypothesis of the mechanism by which the swimbladder is able to regulate the specific gravity of the fish, is not intended as a complete explanation of all the processes involved in gas secretion. It does however offer a chemical and physical explanation of a very controversial matter. The swimbladder of fishes offers unusual opportunities for the study of physiological processes accompanying gas secretion and no doubt new facts may be learned from them of fundamental biological importance.

SUMMARY.

1. The normal gaseous content of the swimbladder of fresh-water fishes near the surface is approximately that of the atmosphere. The composition varies with the species, pressure, temperature, amounts and kinds of dissolved gases, and with the seasons of the year.

2. When fishes are placed in water containing little or no oxygen the oxygen in the swimbladder diminishes; indicating that the swimbladder may act as a reservoir on which the blood may draw for oxygen in times of need. A perch is enabled to go into water of low oxygen content without asphixiation.

3. The effect of increased pressure in the surrounding water is to increase both the percentage of oxygen and carbon dioxide in the swimbladder.

4. If carbon dioxide is increased in the medium in which perch are living, the volume of the fishes is changed and the fishes automatically rise in the water. This response would be of adaptive value, causing the fish to move out of deeper water containing larger amounts of carbon dioxide into the safer zones above.

5. The primary function of the swimbladder of most of the fresh-water fishes is hydrostatic.

6. Perch apparently possess no voluntary muscular control over the size of the swimbladder.

7. Under conditions where high oxygen percentages were found in the swimbladder, a higher tension of the gases existed than in the blood. This indicates an active secretion.

8. Under conditions where fishes are not changing their depth rapidly, the gases in the swimbladder are probably kept constant by simple diffusion of gases from the blood.

9. A "rete mirabile" partially surrounds the walls of the swimbladder and furnishes a rich supply of blood. It is apparently the means by which the gases are transferred from the blood to the swimbladder.

10. The mechanism by which gas is secreted into the swimbladder can apparently be explained on a chemical and physical basis. The writer's experiments show that the hydrogen ion concentration of the swimbladder gland is increased by external stimulation. This indicates the secretion of a substance by

the gland which may aid in the secretion of gases into the swimbladder. The apparent secretion of oxygen is believed to be brought about by (*a*) an increased flow of blood because of the dilatation of the capillaries and (*b*) an increased tension of the oxygen due to the local dissociation of oxygen from oxy-hemoglobin.

11. The swimbladder is a mechanism which enables the fish to actively maintain its stability in the midst of changing external conditions.

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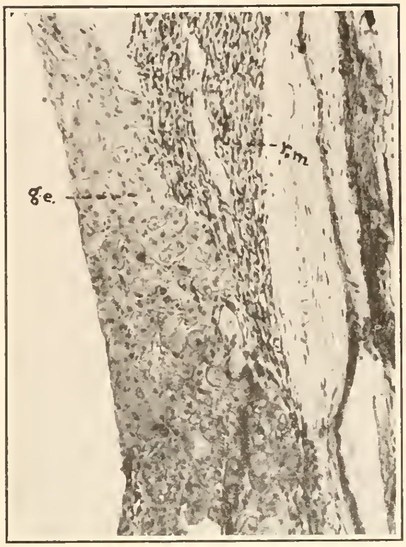
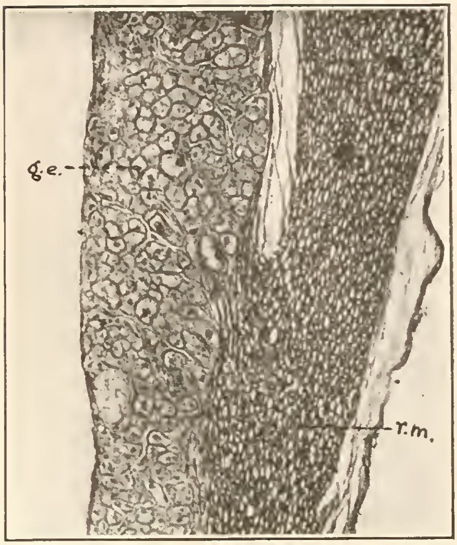
PLATE I.

Photo-micrographs of the swimbladder gland showing the glandular epithelium, *g.e.*; and the rete mirabile, *r.m.* From perch.





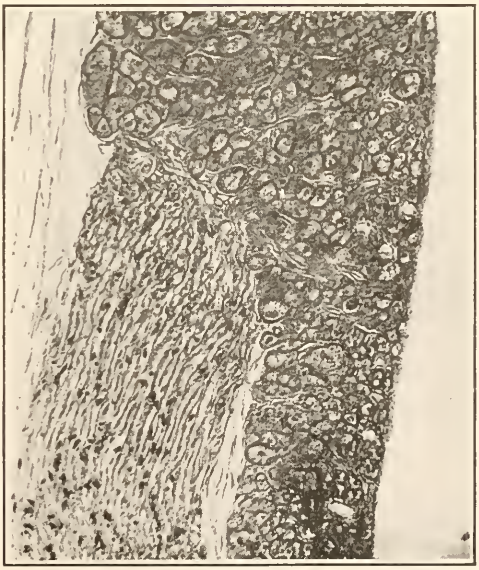


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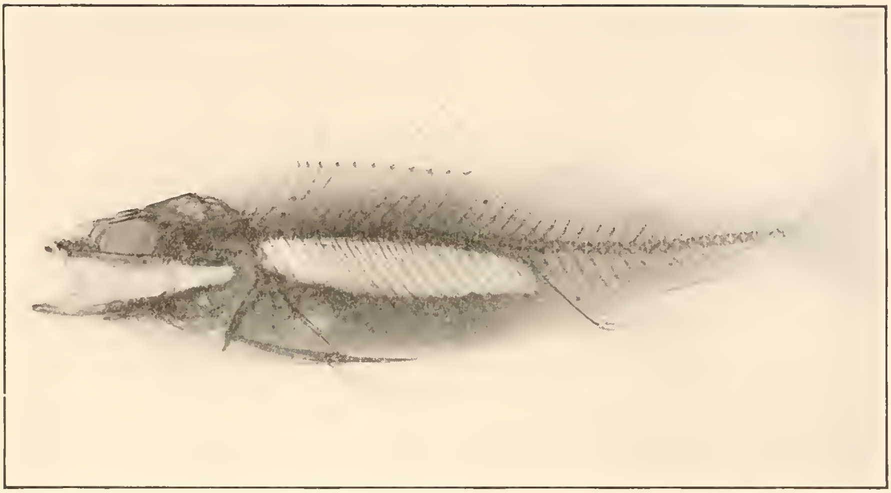
Plate II.

X-Ray of Yellow Perch.
X-Ray of Largemouth Black Bass.



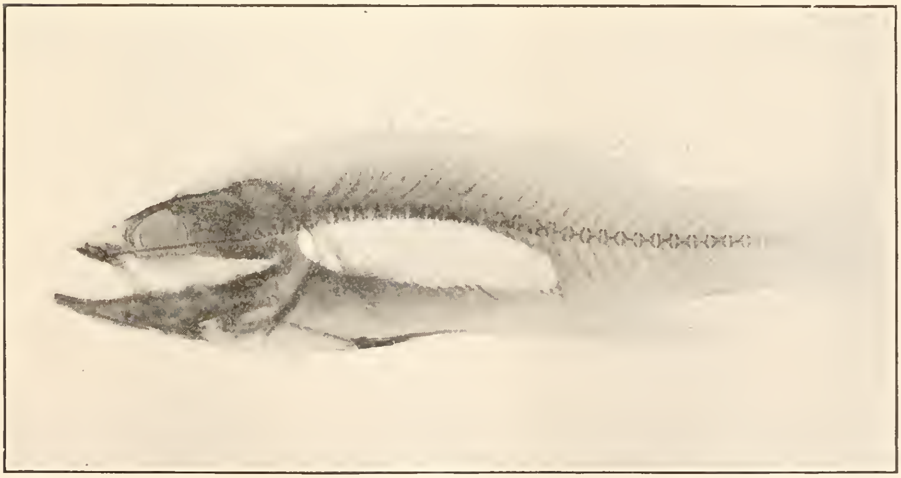


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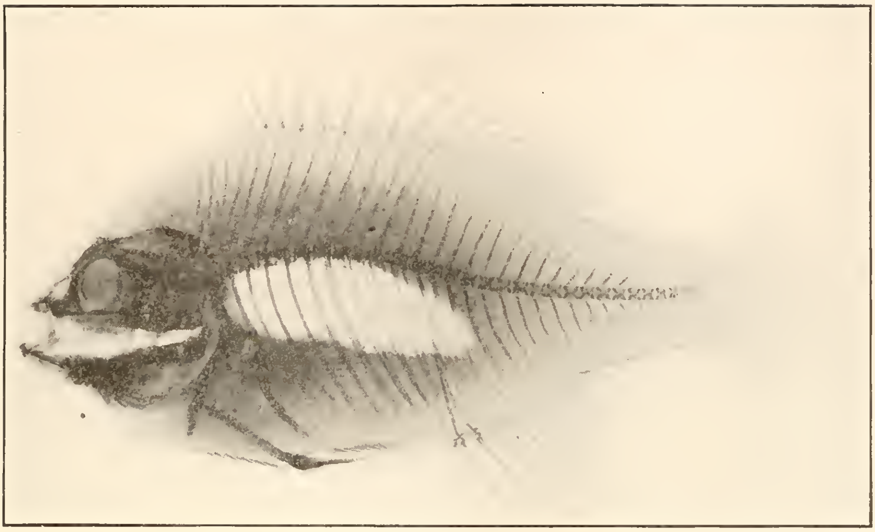
PLATE III.

X-Ray of Sunfish.
X-Ray of Blue-gill.



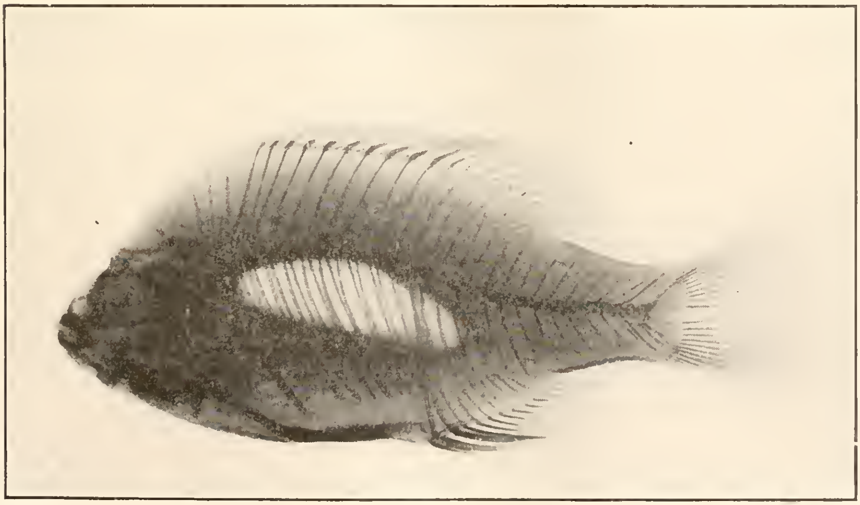


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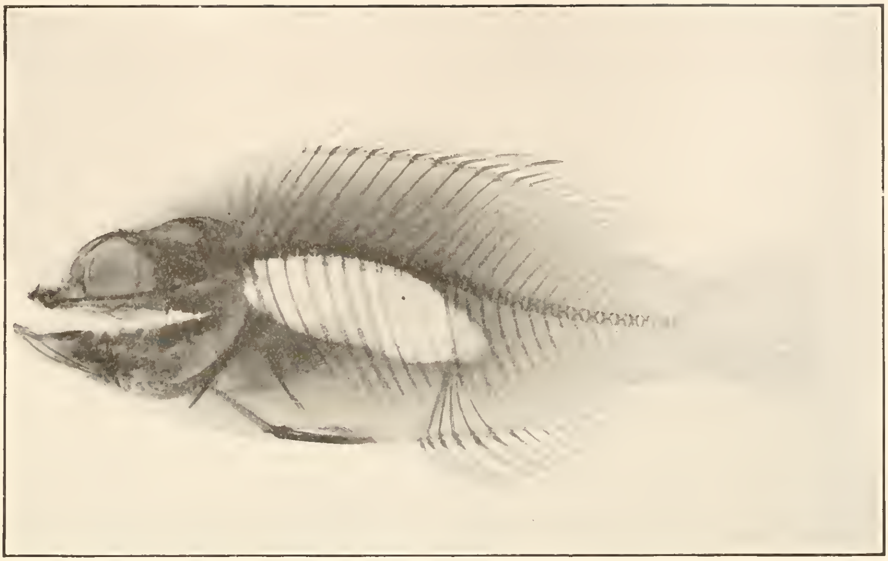
PLATE IV.

X-Ray of Rock-Bass.
X-Ray of Bull-head.



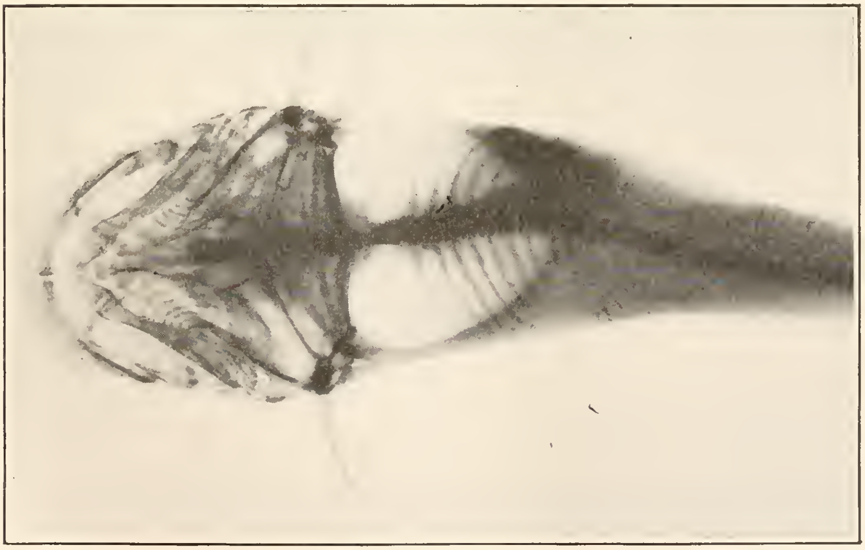


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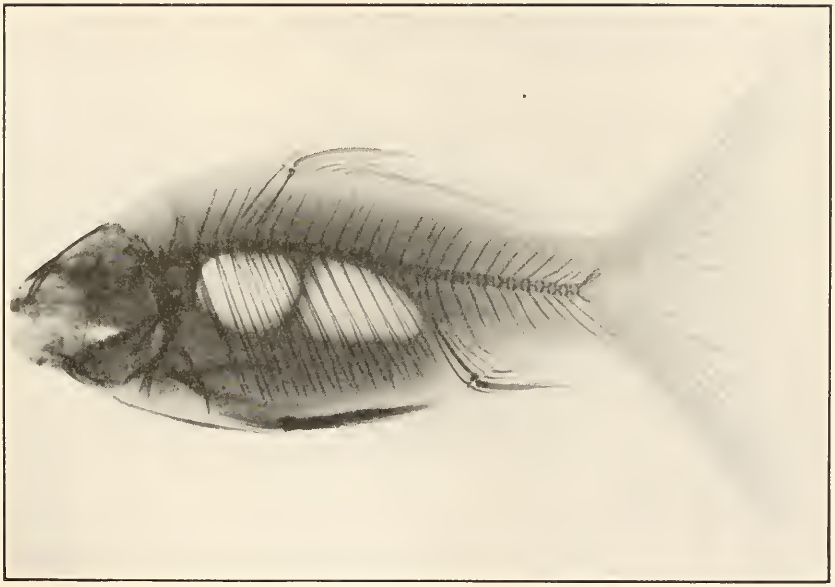
Plate V.

X-Ray of Gold-fish.



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BIOLOGICAL BULLETIN

THE GROWTH OF MARINE ANIMALS ON SUBMERGED METALS.

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In the course of some experiments on the fouling of the bottoms of metal ships, it became apparent that there is a great difference in the ease with which marine animals grow on various metals. To determine the character of this growth, metal plates were suspended for the summer months in a floating wooden frame in the Eel Pond at Woods Hole, Mass. The Eel Pond is a shallow body of salt water almost land-locked, but with a strong tidal circulation and with an abundant marine fauna. The plates were set out early in July and were taken in at the end of August or early in September. In the first year a number of common metals were tested as well as metals covered with various paints. In the second year, beside metals and paints, a number of metal couples were tried In the third and final year only metals and metal couples were used. The present paper has to do merely with metals and metal couples. The work was done at the Marine Biological Laboratory, at Woods Hole, Mass., to the director, Dr. F. R. Lillie, and the officers of which I am under obligations for many courtesies.

The metals used in these tests were the common commercial metals aluminum, zinc, iron, tin, lead, and copper. They were suspended in a wooden frame so as to be in solid contact with nothing but wood, no metal touching them. Each plate was square, measuring about 15 cm. on a side, and was composed of either one metal or a pair of metals doubly overlapped and hammered together, thus making a seam through the middle of the plate. The plates were suspended vertically so that their upper edges were about 20 cm. under water.

Animals soon appeared on many of the plates and in some instances grew with great luxuriance. In no case, however, was the growth so rich as on iron plates covered with certain non-poisonous paints. After six weeks' submergence such plates were often covered with a growth of animals two centimeters thick and so dense that the surface of the plate could nowhere be seen. Thus of all the metals tested, none were so favorable for growth as certain painted surfaces.

The animals that were found on the plates were five common sessile species. Two of these were bryozoans, *Bugula turrita* (Desor) and *Membranipora pilosa* (Linn.), two were tunicates, *Molgula manhattensis* (Dekay) and *Botryllus schlosseri* (Pallas), and one was the common barnacle, *Balanus eburneus* Gould. It is remarkable that the plates were without significant vegetable growth.

The plates exposed represented 36 different habitats: 6 on different single metals and 30 on the 15 possible couples. Of these 36 habitats, 30 were occupied by animals.

Bugula was the most generally present. On all the plates where animal growth occurred at all *Bugula* was found. The fact that *Bugula* is a colonial animal and that the colony is attached to the substrate by a very small stalk explains how it could grow from a surface on which another animal in consequence of more intimate relations to the poisonous substrate could not gain a footing. In the 30 situations occupied by *Bugula* the colonies showed great differences in size and in vigor. The largest colonies on plates that had been in the water 53 days measured 2.3 cm. in height.

Botryllus is the second animal in frequency of occurrence, having been found 28 times in a possible 36. The largest colonies on plates that had been 53 days in the water measured about 5 cm. in diameter.

Membranipora, the third animal, occurred 27 times in a possible 36. The flat colonies of this bryozoan were usually almost circular in outline and the largest formed after 53 days' immersion measured 2.4 cm. in diameter.

Molgula and *Balanus* each occurred 26 times in a possible 36. After a submergence of 53 days the largest *Molgula* measured about 1.5 cm. in diameter and the largest *Balanus* had a diameter of about one centimeter and a height of about half that amount.

The relations of the animals to the various metals can best be considered by taking the single metals first. The conditions of these metals and the kinds of growth that they harbored are indicated in Table I. and on Plate I. All the metals were acted on by the seawater; Al, Zn, Sn, and Pb were slightly corroded, Fe was rusted, and Cu was coated green. A general growth of all five animals to a maximum extent occurred on Al, Fe, and Pb, and a similar growth though to less extent on Sn. Zn carried only a very small amount of *Bugula* and Cu was without any growth at all. In general the metals fall into two groups, Cu and Zn with practically no animals upon them and the other four metals supporting large growths of this kind.

TABLE I.

THE SIX COMMON METALS USED IN THESE EXPERIMENTS ARRANGED IN THE ORDER OF THEIR SOLUTION PRESSURES (ELECTROMOTIVE FORCES).

The effect of the seawater is indicated under the heading State of metal. When all five animals are present the term general is used in the table; when fewer are present they are designated by their generic names. A rough estimate of the amount of growth on the plates is indicated by numbers with 10 as the maximum.

Metal.	Al.	Zn.	Fe.	Sn.	Pb.	Cu.
Solution pressure in volts (H scale)	−1.27	−0.76	−0.43	−0.14	−0.13	+0.34
State of metal . .	Locally corroded	Slightly corroded	Rusted	Slightly corroded	Slightly corroded	Green coating
Animals.	General, 10	*Bugula*, 0.2	General, 10	General, 6	General, 10	Absent, 0

Professor A. B. Lamb, who has given me much help with the chemical side of this problem, has pointed out to me that the marine corrosion of the six metals tested is influenced by other factors than simply their solution tendencies. Among these factors are the solubilities of the hydroxides and basic carbonates of these metals in seawater and the tenacity with which films of these substances cling to the metallic surfaces. Aluminum hydroxide is only sparingly soluble in seawater and forms a firmly adherent coating. Zinc hydroxide and basic zinc carbonate are more soluble and cling less tenaciously. Hence Al will not corrode in seawater so quickly as Zn does. For similar reasons Sn and Pb, though having higher solution pressures than Cu, will actually be less corroded than Cu.

Reasoning from this standpoint, the relative corrosive capacity in seawater of the six metals used in these tests will be in the approximate order Zn, Al, Fe, Cu, Pb, and Sn.

The poisonous effects of these metals on marine animals will depend upon the intrinsic toxicity of their ions, relatively high for all heavy metals, and the solubilities of their hydroxides and basic carbonates in seawater. These solubilities in the case of Fe, Pb, Sn, and Al are in amounts inappreciable; in other words, these metals in seawater are not surrounded by a layer of poisonous ions and hence animals may grow upon them. In the case of Zn and Cu, on the other hand, the corresponding compounds are appreciably soluble in seawater and the poisons thus liberated prevent the growth of animals upon these metals. Thus the presence or absence of poisonous ions or compounds is what determines whether a given metal will be covered with animal growth or not.

Table II.

Couples between Al and the Five Other Metals, all of Which Stand below Al in the Electromotive Series.

Coupled Metal.	Zn.	Fe.	Sn.	Pb.	Cu.
State of Al	Uncorroded	Many lumps of whitish corrosion	Very many lumps of whitish corrosion	Covered with whitish corrosion	Covered with whitish corrosion
Animals . .	General, 10	General, 7	*Botryllus*, *Bugula*, 6	General, 7	General, 6

The animals are indicated as in Table I.

In the metallic couples the metal with the highest solution pressure was Al. All the metals with which it was combined should, therefore, render it active. So far as its solution pressure is concerned, it is so near the next metal in the series, Zn, that it remained, when combined with this metal in seawater, practically uncorroded. In all other couples corrosion was evident and increased in amount as the series was passed over from Fe to Cu (Table II.). Since, however, the products of this corrosion are practically insoluble, a general growth of animals in fair amounts occurred on all the Al members of couples excepting one. In this couple, Al and Sn, the Al member carried only *Botryllus* and *Bugula,* though these were present in considerable amounts. This limitation may have been due to poisonous Sn ions which apparently checked growth in another instance to be referred to later (Fe).

The second metal to be considered is Zn. This stands below Al in the electromotive series, but above Fe, Sn, Pb, and Cu. When combined with Al, it remained uncorroded in seawater (Table III. and Plate II.). With Fe and Sn it corroded slightly and with Pb and Cu much more. A small amount of *Bugula* grew on it when it was combined with Al, where it must have been relatively inactive. In all other combinations animals were absent probably because its activity resulted in the formation of an abundance of Zn ions or soluble Zn compounds.

Table III.

Couples between Zn and the Five Other Metals, One of Which, Al. Stands above Zn, and Four of Which, Fe, Sn, Pb, and Cu, Stand below Zn in the Electromotive Series.

Coupled Metal.	Al.	Fe.	Sn.	Pb.	Cu.
State of Zn.	Uncorroded	Slightly corroded	Slightly corroded	Corroded	Corroded
Animals.	*Bugula*, 2	Absent, 0	Absent, 0	Absent, 0	Absent, 0

The animals are indicated as in Table I.

The third metal is Fe, which stands below Al and Zn and above Sn, Pb, and Cu in the electromotive series. When combined with Al and with Zn, it was not much acted on by seawater (Table IV.), but in combination with Sn, Pb, and Cu it rusted freely.

Table IV.

Couples between Fe and the Five Other Metals, Two of Which, Al and Zn, Stand above Fe, and Three of Which, Sn, Pb, and Cu, Stand below Fe in the Electromotive Series.

Coupled Metal.	Al.	Zn.	Sn.	Pb.	Cu.
State of Fe. .	Gray and granular	Gray and granular	Rusted	Rusted	Rusted
Animals.	General, 10	*Botryllus, Bugula, Membranipora*, 3	General, 2	General, 5	General, 8

The animals are indicated as in Table I.

In the inactive condition, when coupled with Al, a general vigorous growth of animals took place (Plate III.), and the same would probably have occurred in the Zn couple had it not been for the poisonous Zn ions or compounds liberated by the Zn half-plate. When paired with Sn, Pb, and Cu, the growth was general, though often not large in amount. The occasional small quantities were probably due to the physical difficulty presented to the animals of maintaining a foothold on a plate that was continually sloughing its outer layer.

The fourth and fifth metals are Sn and Pb. In the electromotive series Sn stands below Al, Zn, and Fe and above Pb and Cu, and Pb stands above only Cu. The corrosion of Sn and of Pb in seawater follows expectancy in that Sn corrodes only when it is combined with Pb and Cu, and Pb only when it is combined with Cu (Tables V. and VI.). Since in all the combinations the Sn and the Pb are either inactive or give rise to products almost insoluble and hence not ionic, it follows that a general growth of animals is to be expected on Sn and Pb in all combinations, and such appears to be the case (Tables V. and VI. and Plate IV.).

TABLE V.

COUPLES BETWEEN SN AND THE FIVE OTHER METALS, THREE OF WHICH, AL, ZN, AND FE, STAND ABOVE SN, AND TWO OF WHICH, PB AND CU, STAND BELOW SN IN THE ELECTROMOTIVE SERIES.

Coupled Metal.	Al.	Zn.	Fe.	Pb.	Cu.
State of Sn.....	Uncorroded	Uncorroded	Uncorroded	Corroded	Corroded
Animals.......	General, 7	General, 3	General, 5	General, 6	General, 8

The animals are indicated as in Table I.

TABLE VI.

COUPLES BETWEEN PB AND THE FIVE OTHER METALS, FOUR OF WHICH, AL, ZN, FE, AND SN, STAND ABOVE PB, AND ONE OF WHICH, CU, STANDS BELOW PB IN THE ELECTROMOTIVE SERIES.

Coupled Metal.	Al.	Zn.	Fe.	Sn.	Cu.
State of Pb.....	Dark smooth	Dark smooth	Dark smooth	Dark smooth	Whitish coating
Animals.......	General, 10	General, 5	General, 10	General, 9	General, 7

The animals are indicated as in Table I.

In both instances the relative small amount of growth when Sn and Pb were combined with Zn is probably due to the Zn ions or compounds from the neighboring Zn half-plate rather than to the Sn and Pb themselves.

The last metal in the set is Cu, which is below the other five in the electromotive series. In combination with Al, Zn, Fe, or Sn, the half-plate of Cu in seawater remained bright and uncorroded as was to be expected. When combined with Pb, however, it acquired a green coating like that which it developed when, as an isolated single metal, it was exposed to seawater. The solution pressure of Pb is so near that of Cu that probably the Pb is unable to protect the Cu in the way that the other metals do, and hence when it is combined with Pb, Cu corrodes (Table VII.).

TABLE VII.

COUPLES BETWEEN CU AND THE FIVE OTHER METALS, ALL OF WHICH STAND ABOVE CU IN THE ELECTROMOTIVE SERIES.

Coupled Metal.	Al.	Zn.	Fe.	Sn.	Pb.
State of Cu....	Uncorroded	Uncorroded	Uncorroded	Uncorroded	Green coating
Animals.......	General, 10	General, 5	General, 5	General, 3	Absent, 0

The animals are indicated as in Table I.

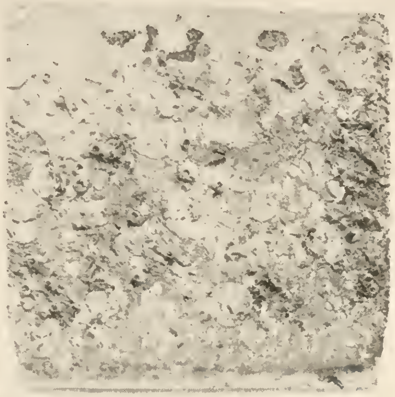
Although Cu, when immersed by itself in seawater, is absolutely free of animal growth, it will support a maximum general growth when rendered inactive by Al. This general growth is also present when the Cu is united with Zn, with Fe, and with Sn, but the quantity falls off as one passes down the series from Al to Sn (Plate V.). Cu has long been known to be most poisonous to the lower organisms and the Cu ions liberated from this metal, when it is immersed alone in seawater, are without doubt the occasion of this quality. It is interesting to observe that in the Cu couples used in these tests the animal growth was at a maximum only with Al and diminished in sequence with Zn, Fe, Sn till it reached nothing with Pb. The orderly diminution in the organisms of different Cu couples just pointed out probably indicates that the Cu was completely inactive only when paired with Al, and that when it was paired with the other metals lower in the electromotive series a very slight but increasing activity appeared, which, though extremely small, was sufficient to check growth. The Cu series also illustrates a historical discovery made by Sir Humphrey Davy, who in the early days of electrochemistry showed that copper-covered ships'-bottoms could be kept from corroding by coupling the Cu with Fe, but that under such circumstances organisms grew upon the Cu in great abundance and thus defeated the object for which the Cu had been applied. It is not impossible, however, that in seaside laboratories and aquaria, where it may be convenient to conduct seawater through copper pipes, the deleterious effect of the Cu can be overcome, at least for short distances, by combining it with some metal high in the electromotive series, such, for instance, as Al.

The observations contained in this paper lead to the conclusion that marine animals will grow upon any heavy metal, provided that metal does not liberate ions or soluble compounds. The ions and soluble compounds of the heavy metals are usually extremely poisonous and where they are liberated freely from a metallic surface that surface is protected against organic growth. Such seems to be the case with Zn and Cu. With Al, Fe, Sn, and Pb the products of marine corrosion are essentially insoluble and hence organisms grow upon these metals in the sea. By coupling Cu with members higher in the electromotive series, this metal can be rendered chemically inactive in seawater and, under such circumstances, animals will grow freely upon it. Zn in this respect is much less easily controlled, for it lies high in the electromotive series and consequently it is not open to the electrochemical protection that Cu is. Its compounds, moreover, are relatively freely soluble and thus become very effective in checking the growth of animals.

Plate I.

Photographs of plates, 15 cm. square, of six different metals which, after a submergence of about six weeks in seawater, were more or less covered by organic growth: 1, aluminum; 2, zinc; 3, iron; 4, tin; 5, lead; 6, copper. For these and the succeeding photographs the author is indebted to Mr. George Nelson.

Al



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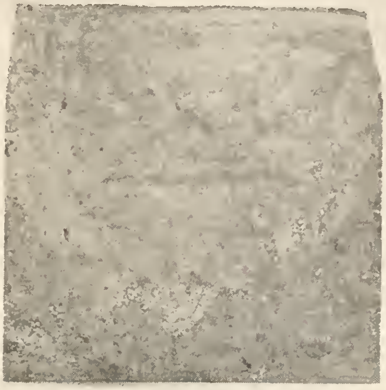
Zn



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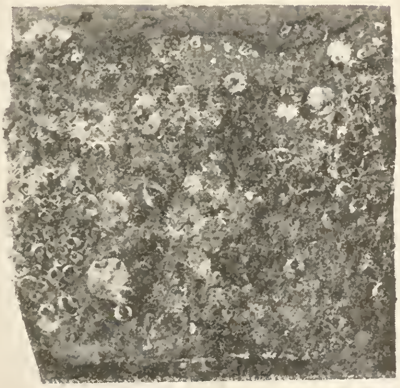
Fe



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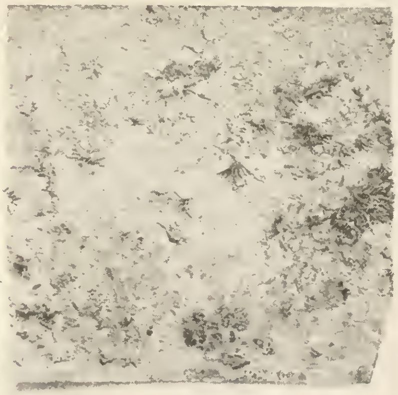
Sn



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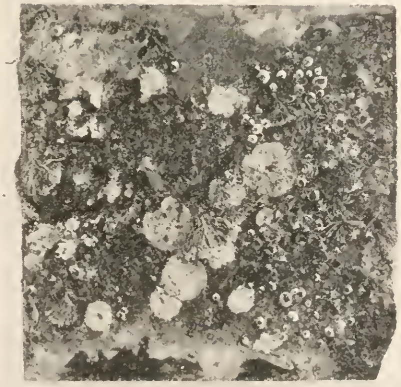
Pb



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Cu

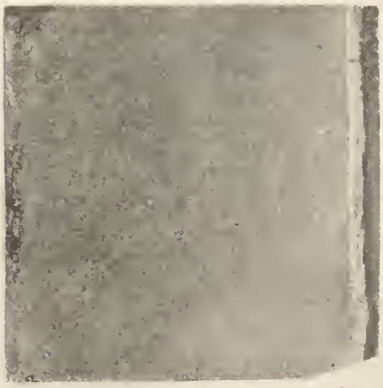

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Plate II.

Photographs of metallic couples in the form of plates, 15 cm. square, more or less covered by organic growth after a submergence of about six weeks in seawater. The series illustrates zinc combined with five other metals as indicated by the symbols on the plate.

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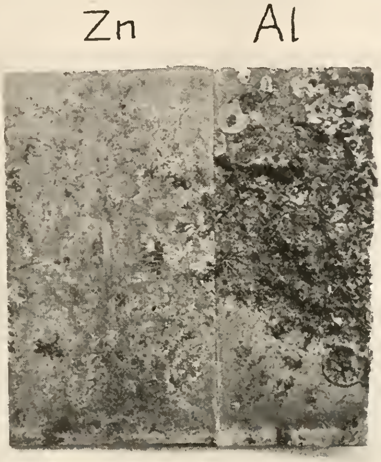

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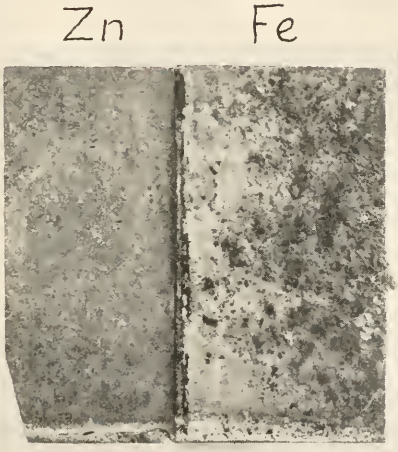

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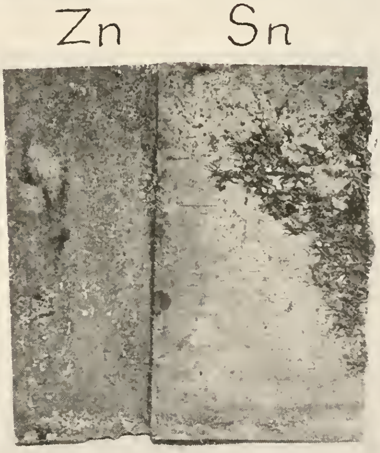

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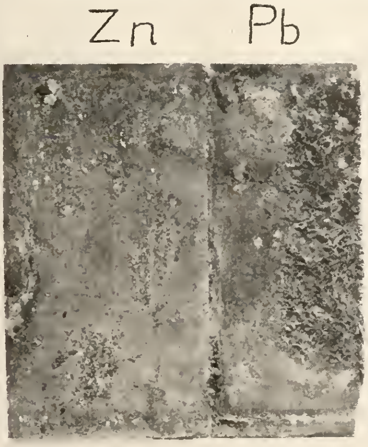

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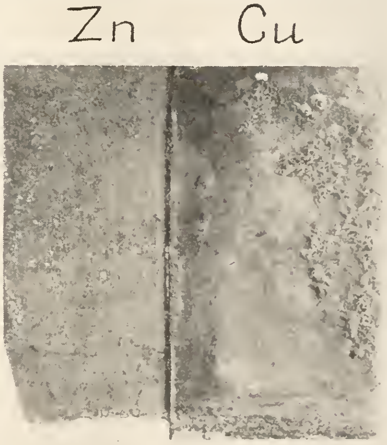
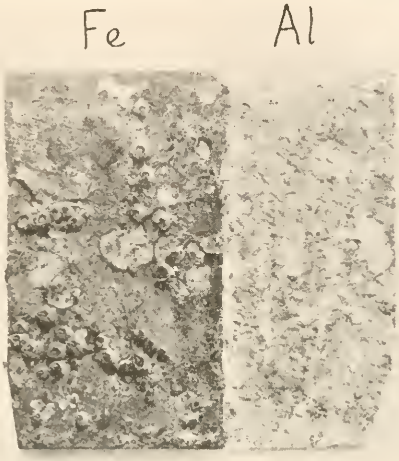


Plate III.

Photographs of metallic couples in the form of plates, 15 cm. square, more or less covered by organic growth after a submergence of about six weeks in seawater. The series illustrates iron combined with five other metals as indicated by the symbols on the plate.



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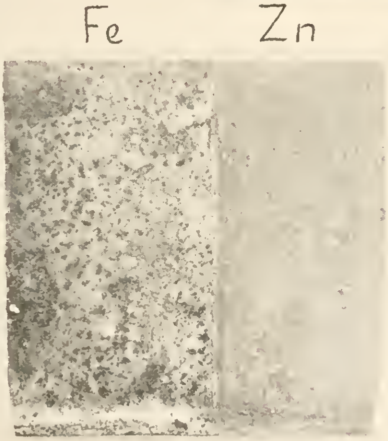

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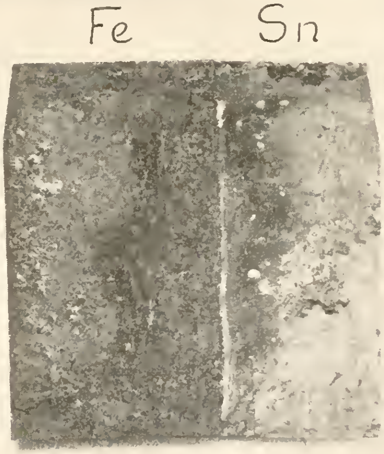

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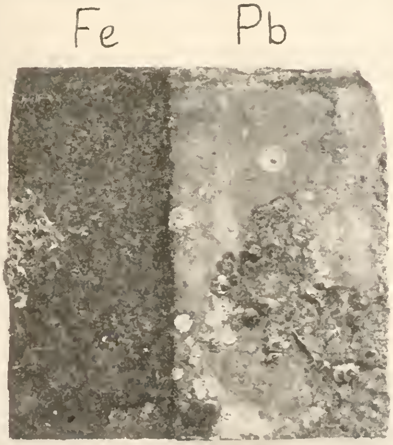

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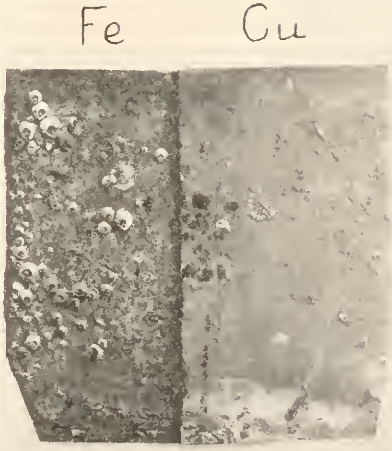

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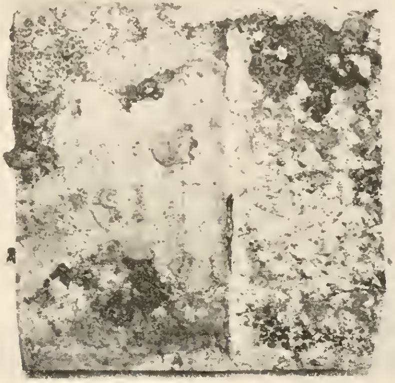
G. H. PARKER.

PLATE IV.

Photographs of metallic couples in the form of plates, 15 cm. square, more or less covered by organic growth after a submergence of about six weeks in seawater. The series illustrates tin combined with five other metals as indicated by the symbols on the plate.



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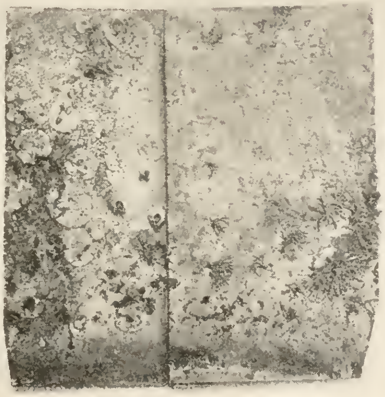

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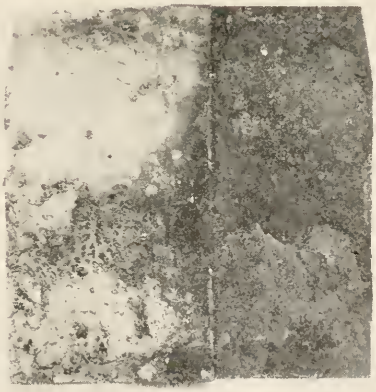

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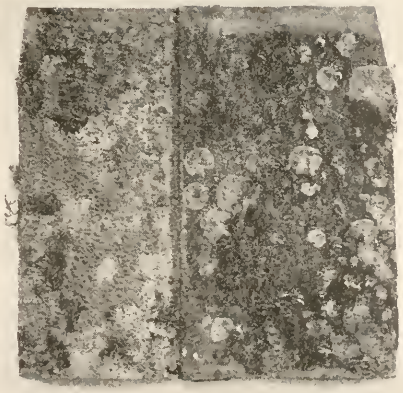

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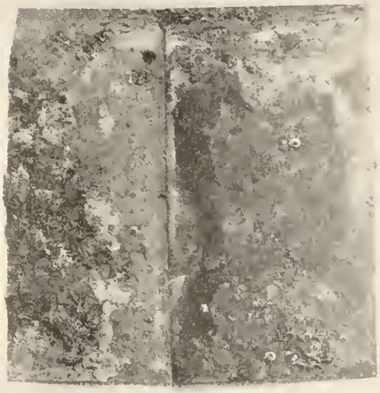

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PLATE V.

Photographs of metallic couples in the form of plates, 15 cm. square, more or less covered by organic growth after a submergence of about six weeks in seawater. The series illustrates copper combined with five other metals as indicated by the symbols on the plate.

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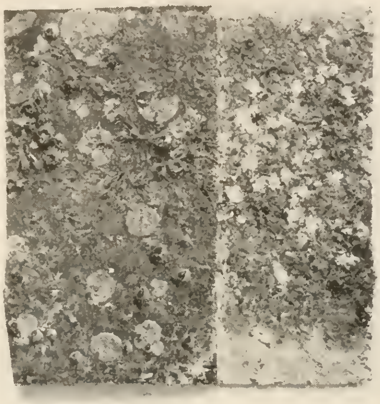

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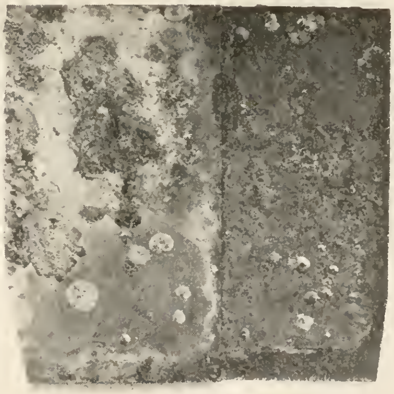

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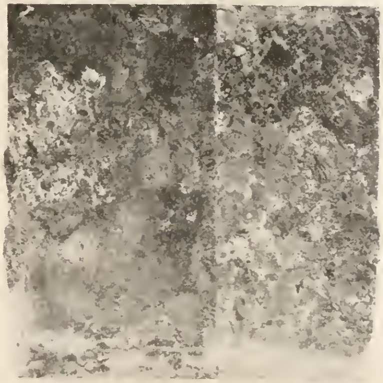

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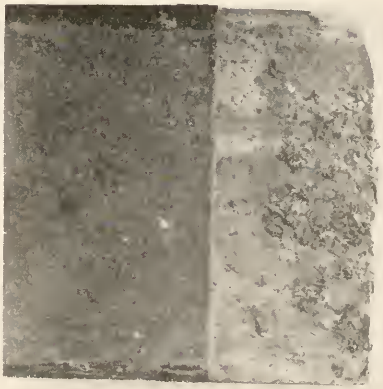

DEVELOPMENT OF THE COMPOUND EYE OF *DROSOPHILA MELANOGASTER* AND ITS BAR-EYED MUTANT.[1]

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University of Georgia, Athens.

A histological examination of the compound eye of *Drosophila melanogaster* Meig., and its bar-eyed mutant, was made to determine its chronological development. The origin of this mutant is known (Tice, 1914). Its germinal behavior has been shown to be sex linked (Tice, 1914). Its major and minor fluctuations in the homozygous condition have been given an exact expression by Zeleny and Mattoon (1915), May (1917), and Zeleny (1917), while the rôles of the various environmental factors have been evaluated by Seyster (1909) and Krafka (1919–20).

The number of facets that are to form the compound eye of the bar-eyed mutant is determined by the temperature at which the larva passes a specific stage in development. This period is definitely established as the third day of larval life when the temperature is 27° C. (Krafka, 1920).

The present paper establishes the condition of the imaginal disks at that time and describes the subsequent changes that take place in the growth and differentiation of the ommatidia through the late larval and the pupal periods.

Entire larvæ, pupæ, and adults were fixed in Bouin's picroformol solution, representing developmental material of 24-hour intervals over the entire life history. Records were kept of the matings, fixation of material, and temperature. The latter ranged from 25° C. to 28° C. Hot water and hot Gilsson's fluid were used with some success. For cell detail, the brain, cord, and imaginal disks were dissected out and fixed in Flemming's strong solution.[2]

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[1] Contribution from the Zoölogical Laboratory of the University of Georgia, No. 5.

[2] Acknowledgments are due Mr. Shelley C. Davis for drawing Fig. 8.

Weismann (1864) was the first to show the relation of the imaginal disks to adult structure. The development of these disks can be readily followed in *Drosophila* either by direct dissection or by serial section.

The primordia of the imaginal disks of the compound eye arise as invaginations of the so-called pharynx, with which they retain their connection by a delicate membrane. They can be found as late as the two-day larva, anterior to and independent of the brain or supra-esophageal ganglia (Fig. 1).

The posterior migration of these disks results in their fusion with the anterior aspects of these ganglia. This connection is of a membranous character and the disks and brain may be readily removed together in dissection. This condition is met within the three-day larva (Fig. 2). As determined by temperature experiments on the bar-eyed mutant, this is the critical period in facet formation.

That the rudiments of the ommatidia are fixed at this time is shown by the peculiar arrangement of cells in the imaginal disks of the four-day larva. Four terminal cells and six basal cells, arranged around a deeply staining axis, form a cylindrical unit that is repeated over the entire inner surface of the imaginal disk (Figs. 3 and 4).

Pupation occurs on the fifth day of larval life. The imaginal disks take their position as a part of the body wall of the pupa.

On the sixth day the various parts of the dioptric apparatus are clearly recognizable as such. The rudimentary ommatidia are spherical in shape, with four terminal pseudocone cells and six retinulæ cells arranged around a rhabdome. The ommatidia are supported by a double row of pyramidal cells, one basal and the other peripheral. The peripheral cells underlie the short optic bristles (Figs. 5 and 6).

Material slightly more advanced shows an elongation of the retinulæ cells and the rhabdome. The outer surface of the four pseudocone cells is convex and a very thin cuticula has formed. This separates from the ommatidia in the process of fixation in the same manner as the corneal facets (Fig. 7).

By continued elongation the rhabdome and the retinulæ cells reach their ultimate length. The four pseudocone cells are still to be found in the vesicle formed by the supporting cells on the seventh day. They lie immediately below the corneal facets which have now become more decidedly plano-convex. No so-called optic cup is present at this time, but an examination of the living fly shows the beginning of the formation of pigment (Figs. 8 and 9).

The eye is completed on the eighth day by the cupping of the pseudocone, leaving the four pseudocone nuclei surrounding the end of the rhabdome at the apex of the cone (Fig. 10).

The adult leaves the pupal case on the ninth day.

This brief résumé of the ontogeny of the compound eye presents some interesting problems in correlative development. Kopeč, 1922, by the removal of the ganglia of the caterpillars of *Lymantria dispar* L., after their last moult, showed an entire independence of the development of the optic apparatus and the brain. In *Drosophila* it has been shown, however, that the ultimate structure of the eye is determined very early in larval life. The subsequent removal of the brain would not be expected to influence further development. The present study shows the forerunners of the ommatidia to be structurally present on the fourth day of larval life.

That the nervous system and the dioptric system are closely correlated in their development is further shown by the striking reduction in the size of the optic ganglion in the bar-eyed mutant. When compared with that of the full-eyed fly it is seen to be less than half as great in diameter (Fig. 11).

SUMMARY.

1. The cell groups, representing the primordia of the ommatidia of the compound eye of *Drosophila melanogaster,* have been found in the four-day larva.

2. The fusion of the imaginal disks and the supra-esophageal ganglia take place on the third day of larval life.

3. There is a correlation between the development of the nervous system and the dioptric apparatus, as shown by the reduction in the size of the optic tract in the bar-eyed mutant.

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EXPLANATION OF FIGURES.

Plate I.

Fig. 1. Frontal section through the anterior end of a two-day larva. The imaginal disks (*I*) of the compound eye are shown attached to the so-called pharynx and are far removed from the supra-esophageal ganglia (*G*). 300 ×.

Fig. 2. Sagittal section through the anterior end of a three-day larva. The imaginal disk (*I*) is shown in union with the brain. 300 ×.

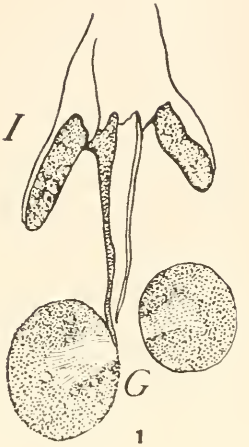
Fig. 3. Section through the imaginal disk of a four-day larva. The cells are arranged around a central axis, while the groups correspond to the ommatidial arrangement in the adult. 1300 ×.

Fig. 4. Section through the imaginal disk of another four-day larva. The rudiments of the ommatidia appear as rosettes of four or six cells in cross-section. 2100 ×.

Fig. 5. Cross-section of the head of a two-day pupa (sixth day), showing the compound eye in place and the ommatidia present as such. The latter are connected to the optic tract by nerve fibres. 300 ×.

Fig. 6. The same ommatidia greatly magnified. The rhabdome, six retinulæ cells, the supporting cells, and the bristles are all clearly shown. The figures at the side represent cross-sections at two different levels. 1300 ×.

Fig. 7. Longitudinal sections through the ommatidia of a pupa slightly older than that of Fig. 6. 1300 ×.



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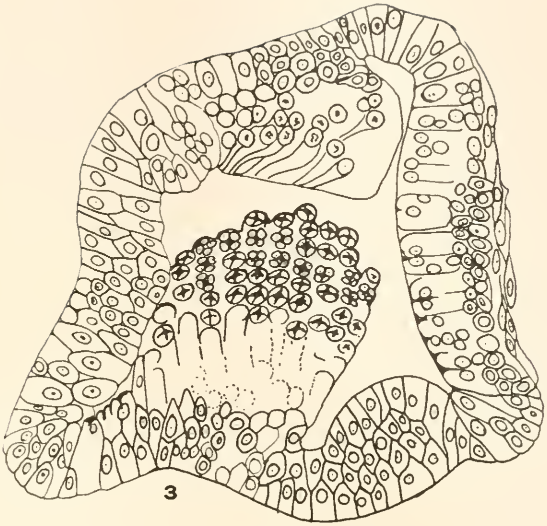

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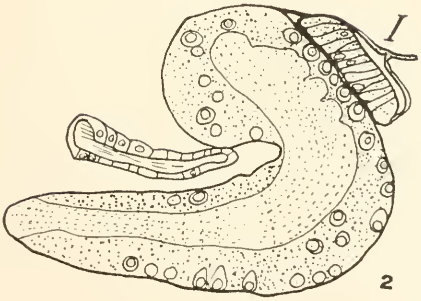

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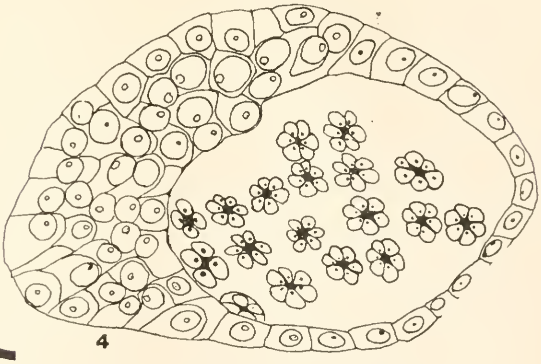

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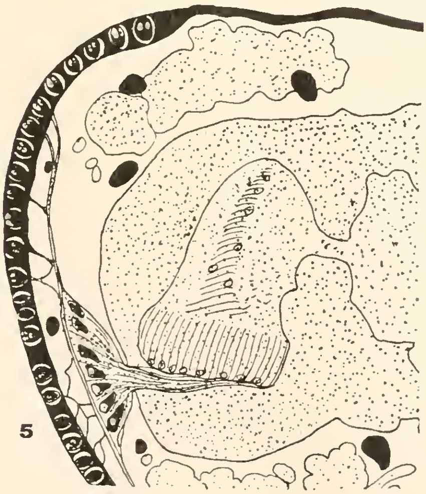

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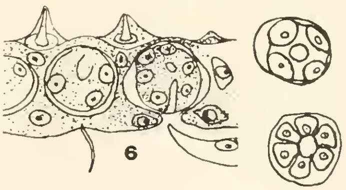

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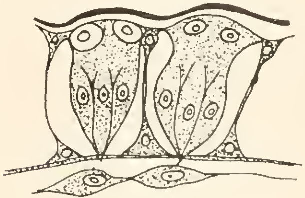

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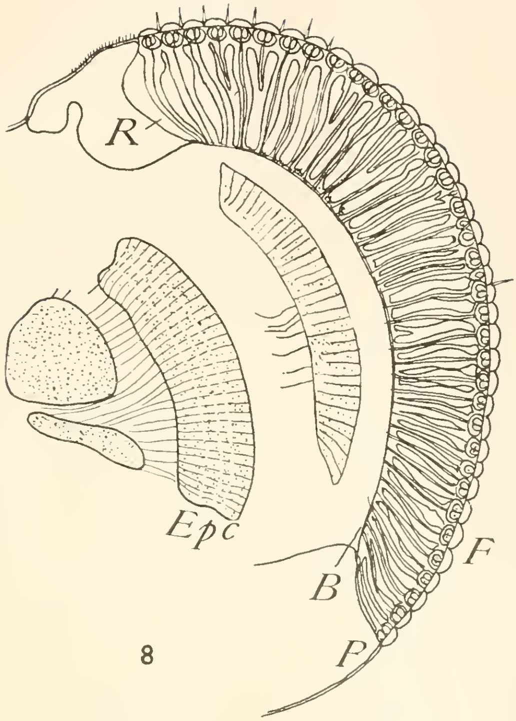
Plate II.

Fig. 8. Cross-section of the head of a three-day pupa (seventh day). The facets (*F*), pseudocone cells (*P*), rods or rhabdome (*R*), and the basement membrane are practically complete. *Epc* is the epiopticon. 300 ×.

Fig. 9. Enlarged outline drawing of two ommatidia in this stage. 1300 ×.

Fig. 10. Outline drawing of two ommatidia of the adult. The figures at the side are cross-sections of an ommatidium at various levels. 1300 ×.

Fig. 11. Composite drawing of the head of a bar-eyed mutant (at the left) and a full-eyed wild adult (at the right) to show the striking reduction in the size of the optic tract in the former. 200 ×. *BO* is the optic tract of the mutant, *O* is that of the full-eyed wild stock.



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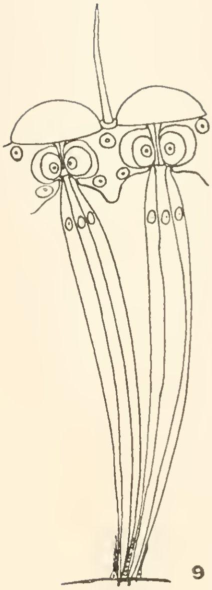

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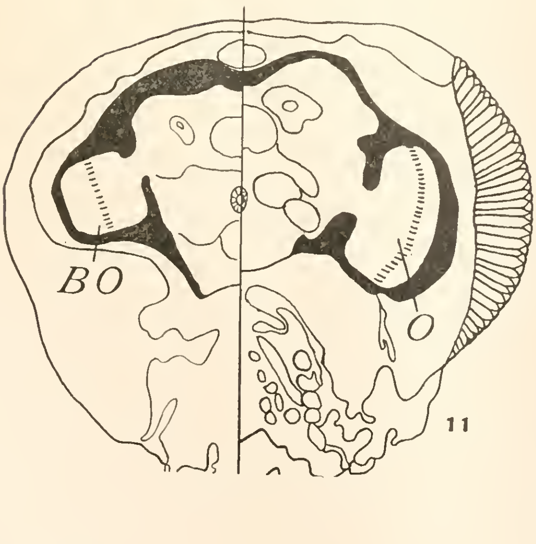

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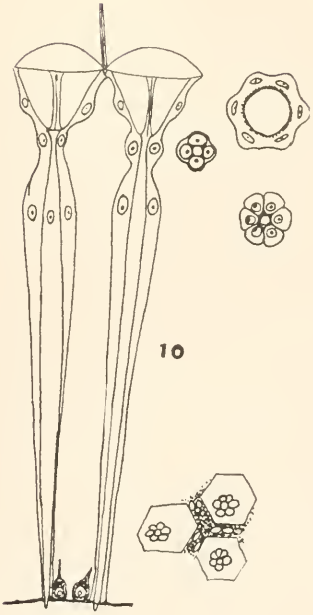

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STUDIES ON *EUGLENAMORPHA HEGNERI* N. G., N. SP., A EUGLENOID FLAGELLATE FOUND IN TADPOLES.

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Introduction.

In a list of *Protozoa* to be found in tadpoles Hegner ('22) mentions the discovery of a *Euglena*-like flagellate with three flagella together with other euglenoid forms. The writer had also discovered the three-flagellated species in tadpoles previous to the publication of Hegner's note and presented a brief account of its structure and variations at the meeting of the American Society of Zoölogists, December, 1922 (Wenrich, '23). More recently Hegner ('23) has published an account of his observations on this and other euglenoids in tadpoles. Since no description of this organism has been found in previous literature, the name *Euglenamorpha hegneri* is proposed for it. It is the purpose of this paper to describe this new species and especially its morphological variations.

Materials and Methods.

A few years ago the writer began the collection of material for the study of the intestinal *Protozoa* of American *Amphibia,* giving especial attention to the flagellates. Every opportunity to obtain material was taken and tadpoles were examined when available. As reported by Hegner ('22), tadpoles were found to be abundantly infested with intestinal protozoa and the relation of these to the intestinal forms of the adult *Amphibia* presented itself as an interesting problem. A small pond in the botanical gardens of the University of Pennsylvania harbors a goodly number of bullfrogs and their tadpoles which offered convenient material for the study of this problem, and it was in the intestinal contents from these tadpoles that *Euglenamorpha* was first recognized in June, 1922. Of the 117 bullfrog tadpoles examined during the period from May 31, 1922, to January 19, 1923, 68, or 58 per cent., were found to contain *Euglenamorpha.* Most of these tadpoles were obtained from the pond mentioned above. Tadpoles of *Rana palustris* collected by Dr. C. L. Parmenter and fed on vegetation from the same pond were also infested by this flagellate. I am much indebted to Dr. Parmenter for permission to examine his tadpoles.

During the summer of 1923, 35 tadpoles of *R. palustris* from some collected and reared by Dr. Parmenter were examined and 21 or 60 per cent. contained *Euglenamorpha,* while of 30 bullfrog tadpoles from the pond only 5, or 16⅔ per cent., revealed this flagellate. On the other hand, of 8 tadpoles of *R. clamitans* (?) and 8 of *Hyla versicolor* examined at Woods Hole, Mass., every one contained *Euglenamorpha.*

The colorless variety (described beyond) was frequently found in the bullfrog tadpoles during the summer of 1922, but has not been found in any of the tadpoles of *R. palustris* nor in the tadpoles of any of the species examined in 1923.

An examination of slides made from the rectal contents of bullfrog tadpoles collected from the pond in September, 1919, revealed the presence of *Euglenamorpha* at that time, and it is probable that it has existed there for an indefinite period.

This new flagellate is usually found in the rectum of the host and is commonly more numerous in the middle and posterior regions of that organ than in the anterior portion, which is favored by most of the other entozoic species. Hegner ('23) found it in the small intestine as well as the rectum in a number of cases and active individuals have also been seen in freshly deposited fæces.

The entozoic character of *Euglenamorpha* was established in a number of ways. At first it was considered possible that the organisms had come from the surrounding water. Consequently a number of tadpoles were killed by dropping them into Schaudinn's and Bouin's fixatives. They were then carefully washed in sterile water and examined and found to contain the flagellates. In other cases the tadpoles were opened and the entire digestive tract removed and examined in salt solution without teasing. The flagellates could then be seen swimming actively about in the lumen of the rectum, thus proving their entozoic habit. The hundreds of individuals that were sometimes present in smears made from the rectal contents and fixed and stained also showed that they were obtained from the tadpoles and could not have been derived from an external source. Hegner's ('23) observations also leave no doubt as to the entozoic character of *Euglenamorpha*.

For fixation of smears, Schaudinn's fluid has been constantly employed, although occasionally Bouin's fluid and Flemming's both stronger and weaker fluids, chrom-acetic, and sublimate-acetic were resorted to, but with no apparent advantage. For staining, Heidenhain's iron-alum-hæmatoxylin has been mostly employed, although Delafield's hæmatoxylin and hæmalum have also been used. The first of these has always given the most satisfactory results.

All the figures except 1 to 6 have been drawn with a camera lucida from slides fixed with Schaudinn's fluid and stained with iron-alum-hæmatoxylin. Figures 1 to 6 are drawn from living material on the same scale as the others. All are magnified about 1,100 diameters.

Descriptions and Observations.

A. Diagnoses.

Euglenamorpha, new genus. Diagnosis: characters like those of *Euglena* except for the three to six flagella and the entozoic habit. The *Euglena*-like characters include elongated body, presence of chlorophyll and red stigma, vacuolar and pharyngeal apparatus, nucleus with central caryosome surrounded by chromatin in the form of granules or strands, periplast spirally striate, metabolic activity. The distinguishing characters of the new genus are (1) the presence of three to six flagella and (2) the entozoic habit.

Euglenamorpha hegneri, new species. Since there are two strikingly different although intergrading varieties, it seems desirable to establish the green, three-flagellated kind which Hegner discovered as the type variety and to regard the other one as a variation.

Type form. Diagnosis: *Euglenamorpha* with body, when elongated, generally cylindrical to cigar-shaped, tapering more at the posterior than at the anterior end. Length, 30 to 55 microns, with an average of about 45 microns. Width, 4 to 8 microns, with an average of 5.5 microns. Green chloroplasts, paramylon granules, and red stigma present. The three equal-length flagella are from one-half to two-thirds the length of the body and each has a spindle-shaped swelling on its root in the reservoir adjacent to the stigma. Nucleus, generally compact so as to obscure the caryosome, commonly placed laterally near the middle of the body. Surface striæ fine, numerous, and usually spirally arranged passing from the left over to the right. Habitat, rectum of tadpoles.

Variety *pellucida.* This variety has the body elongately conical, widest a little behind the reservoir, tapering from there gradually to a sharp posterior tip and more abruptly to the rounded anterior end. Size, similar to the type variety, but averaging a little smaller. Colorless or slightly greenish. Flagella mostly four or six, sometimes two, three or five, with no swellings on the roots. Reservoir enlarged, nucleus expanded to width of the body, caryosome conspicuous and often multiple. Surface striæ prominent to absent, generally spirally arranged passing from the right over to the left, although they may have the reverse arrangement or lie parallel with the long axis. Habitat, rectum of tadpoles.

B. The Two Varieties Compared.

When the two different varieties were first recognized their differences were so striking that it was thought that they should go into different species if not into different genera, but further study makes it highly probable that the green (type) form transforms into the colorless (*pellucida*) one. When typical individuals of the two varieties are compared they are found to differ in almost every detail.

The form of the body of the green variety is well illustrated in Figs. 1, 7, 8, and 9. It is more nearly cylindrical or cigar-shaped as contrasted with the conical form of *pellucida* (Figs. 12 to 16). In side view, the anterior end often appears obliquely truncated with a notch which marks the location of the "mouth." There is some variation in the posterior end and conditions as shown in Fig. 20 are occasionally met with. The conical form of *pellucida* is very constant in combination with the other characters of this variety.

The surface striæ of the typical green individuals are fine, numerous, and take a sharply spiral course passing from the left over to the right as shown in Fig. 7. Occasionally they may be parallel to the long axis (Fig. 8) or even pass spirally from right to left (Fig. 9). In contrast the striæ of the pellucid variety usually pass spirally from right to left (Figs. 21, 28, 33) or are parallel to the long axis (Figs. 12 to 16). They may, however, occasionally show the reverse spiral condition typical of the green variety. There is considerable variation in the appearance of these striæ in the pellucid variety, with evidence of their gradual disintegration. They are frequently less numerous (Figs. 12, 16, and 33) and often between these few there are fainter ones (Fig. 13). In other cases the striæ appear to be breaking up (Fig. 15) and they may not show at all. These various conditions indicate that with the transformation of the green variety into the colorless one the striæ become reversed in position and may gradually disintegrate, a set of alternate striæ disappearing first. This latter feature is the reverse of the process of adding new striæ after division in euglenoids, when new ones are interpolated between those carried over from the parent.

The chloroplasts of the green variety are typically rounded disks placed peripherally for the most part, but close enough together to give the impression of a continuous green color. In some of the cultures the chlorophyll became unevenly distributed, often disappearing from one end or the other. In a few individuals it disappeared altogether in the cultures. Deeper in the protoplasm are the smooth, refractive oval granules which appear to be paramylon bodies. Usually from two to three microns long by one and one-half to two microns wide and numbering from a dozen to three dozen (Figs. 1 to 6), they are sometimes much smaller and more numerous especially in individuals which are losing their chlorophyll. In the cultures these granules often persisted for days or weeks after the protoplasm of the individual of which they had been a part had disintegrated. In the fixed and stained individuals the chloroplasts persist as stained bodies (Figs. 7 to 9), while the paramylon granules disappear.

The nuclear conditions in the two varieties are of considerable interest. In the typical green individual the nucleus is compact, the chromatin granules and strands being so close together that they obscure the caryosome (Figs. 7 and 8). The typical nucleus is from one-half to two-thirds the diameter of the body and is laterally placed. In transforming or possibly degenerating individuals the nucleus may expand to conditions approximating those of *pellucida* (Figs. 10, 11, and 20). In the pellucid variety there is a marked tendency for the nucleus to hypertrophy. It expands so as to approximate the diameter of the body and then elongates along the axis. This hypertrophy is accompanied by a multiplication of the caryosome, as many as four having been found in one nucleus (Figs. 12 to 16). Hypertrophy apparently leads to amitotic division of the nucleus (Figs. 32 to 35), which is probably followed by division of the cell body (Fig. 36). Such amitotic stages have not been found in the green variety.

The flagellar situation is somewhat puzzling. In the green form more than three flagella have not been found, although prolonged search has been made. These flagella take their origin from basal granules just behind the reservoir and are separated for their entire length passing through the reservoir, "pharynx," and "mouth" to the exterior. At a point in the reservoir nearest the stigma each flagellar root has a spindle-shaped swelling which stains intensely with hæmatoxylin. These chromatic swellings seem to be the most constant character possessed by the green variety and in the fixed and stained individuals serve readily to distinguish the type from *pellucida,* where they are entirely absent.

In the colorless variety from two to six flagella have been found, but the great majority have either four or six. In a count of two hundred from a single slide 6, or 3 per cent., had two flagella; 7, or 3.5 per cent., had three; 87, or 43.5 per cent., had four; 21, or

10.5 per cent., had five; and 79, or 39.5 per cent., had six. Individuals with two or three flagella (Figs. 36 and 26) may be daughters from a recent division. The significance of the four- and six-flagellated condition is problematical, but will be discussed further on. The two groups differ in size, it was found. A series of four-flagellated individuals were found to range from 32 to 44 microns in length, with an average of 38.25 microns, while a series of six-flagellated individuals averaged 45.6 microns in length, with a range of 42 to 52 microns. The roots of the four or six flagella are usually segregated into two equal groups, with the corresponding groups of basal granules some distance apart in the posterior wall of the reservoir. This condition is probably correlated with the prevalent stages of amitosis found in the nucleus (Figs. 12 to 16 and 32 to 35).

The reservoir is regularly of larger size in *pellucida* than in the type and the absence of other stainable material makes it easier to distinguish the basal granules of the flagella in its posterior wall. No rhizostyles extending from the basal granules toward the nucleus have been found.

The stigma (*s,* Fig. 1), found only in the green variety, has a structure similar to that of other Euglenoids, being a disk of closely aggregated granules placed peripherally in the region of the reservoir adjacent to the swellings on the roots of the flagella. These swellings and the stigma probably have some coördinate function and usually disappear simultaneously in the transformation to *pellucida.* The stigma does not persist in slides fixed and stained by the usual processes, but did persist for a day or two in smears fixed with osmic acid fumes and mounted directly in glycerine jelly.

Another difference between the two varieties is revealed in the cultures where the type variety persisted much more readily than did the colorless one. In certain cultures the green variety went into a resting state where it divided (Figs. 4 to 6). In other cultures they multiplied in the active state. The pellucid variety showed neither of these conditions.

In the abstract already published (Wenrich, '23) it was stated that division stages had not been seen for the green variety except in cultures, and that this fact constituted one of the differences between the two varieties, both mitosis and amitosis having been found in *pellucida*. Since then stages in mitosis have been discovered in the type in fixed and stained preparations, but are rare, having been noted on only two slides out of the many that have been prepared. Amitosis has not been seen in the type, although in those with expanded nuclei some tendency to a division of the caryosome has been observed.

The stages of mitosis for the green variety (Figs. 22 to 25), from the slide where they were the most numerous (they were rare even here), seem to be accompanied by transformation into the pellucid variety. The chlorophyll is reduced and the chromatic swellings on the roots of the flagella seem to disappear gradually. The swellings are well shown in Fig. 22, are much reduced in Fig. 23, appear to be absent in Fig. 24, but traces can be seen in Fig. 25, a later stage. The surface striæ in all four stages are characteristic of the type variety.

TABLE I.

A SUMMARY OF THE COMPARISON BETWEEN THE TWO VARIETIES.

Characters.	Type variety.	Variety *pellucida*.
Body form	Cylindrical or cigar-shaped.	Elongately conical.
Surface striæ	Spiral, from left over to the right.	Spiral, from right over to the left, or longitudinal.
Flagella	Three.	Usually four or six.
Swellings on flagellar roots	Present.	Absent.
Reservoir	Smaller.	Larger.
Color	Usually bright green.	Colorless or slightly greenish.
Nucleus	Usually compact; caryosome obscured.	Expanded; caryosome conspicuous, often multiple.
Results in cultures	Assumed resting state; multiplied; persisted 5 months in one.	Failed to assume resting state, multiply, or to persist.
Divisions	Rare in host; rapid in some cultures.	Rapid in host by both mitosis and amitosis; not in cultures.

The mitotic divisions of the nucleus of *pellucida* (Figs. 27 to 31) differ in no essential respect from those of the type. There seems to be a difference in the case of the flagella, however. A double number of flagella (4 or 6) is already present in *pellucida* and are merely segregated into the two daughter cells, whereas in the type additional flagella seem to develop during division. In Fig. 24 there were two groups of flagellar roots, but only three flagella were seen on the exterior. In Fig. 25 the two groups of flagellar roots were still more plainly visible, but the external portions did not show. The small individual with three flagella and a body profile intermediate between the two varieties (Fig. 26) might well be the product of such a division. In *pellucida* new flagella are probably developed soon after division, since so many of them have the even numbers, four or six. Fig. 36 might represent the product of either mitotic or amitotic division, and its small size and two flagella indicate that it is a daughter of a four-flagellated parent.

A summary of the comparison between these two varieties is given in Table I.

C. Transition Stages.

Although the two varieties just described are so strikingly different in their typical forms, enough transition stages between them have been found to make it almost certain that the green or type variety transforms into the colorless or pellucid one. This transformation probably takes place in more than one way. As described above, the mitotic division of the green variety within the host seems to be accompanied by the loss of the chromatic swellings on the roots of the flagella (Figs. 22 to 25). The presence or absence of these swellings has proved to be the best criterion in fixed and stained individuals for distinguishing between the two varieties. Hence it seems probable that division of the green variety is accompanied by transformation.

Transformation probably takes place directly without division. Stages showing the gradual loss of chlorophyll accompanied by the gradual swelling of the nucleus to the expanded condition characteristic of *pellucida* are not hard to find (Figs. 9 to 11, 17 and 20). While this series of changes may lead to complete degeneration as observed on some slides, in other cases they may lead to the colorless variety. The chromatic swellings may disappear before the chlorophyll has all broken down (Figs. 18, 19, 21) or before the change in the direction of the surface striæ (Fig. 18). In Fig. 18, however, it is seen that one of the three flagellar roots still retains a remnant of its chromatic swelling. Fig. 19 might be a further stage in the transformation with the swellings entirely gone, the striæ changing their direction, but chlorophyll still present. The individual shown in Fig. 20 has the swellings and surface striæ of the green variety, but the chloroplasts have largely disappeared and an extra flagellar root without a swelling shows in the reservoir. The origin of the four-flagellated *pellucida* is possibly indicated.

In one of the cultures (see below) a pellucid individual appeared among the green ones a month after the culture was started. It is probable that it transformed from a green one. Altogether the evidence at hand points to the occasional transformation of the green variety into the colorless one.

C. *Results from Cultures.*

In attempts to cultivate *Euglenamorpha* outside the host two general methods were employed. In one a section of the rectum or a piece of its contents bearing the flagellates was placed in a small stender dish containing the medium. The other method was that of the hanging drop, in which a drop of the medium was placed in the middle of the cover glass, the rectal material added, and the cover then inverted over a depression slide and sealed with vaseline.

In the fifteen dish cultures the following media were tried: ovomucoid, beef bouillon, Sellard's ('11) liquid medium modified by Dr. Martha Bunting ('22), who very kindly prepared the foregoing media; Pringsheim's ('15) solution, Ringer's solution, 0.6 per cent. sodium chloride, equal parts of 1 per cent. sodium chloride and 1 per cent. sodium citrate, sterile pond water, various combinations of these and the use of some of them in combination with 2 per cent. agar. In the eleven hanging drop cultures the abovenamed media were also used except the ovo-mucoid, the beef bouillon, and the agar.

None of the dish cultures served to keep *Euglenamorpha* alive more than a few days and in none of them was there any evidence of multiplication. *Trichomonas* flourished on a mixture of ovomucoid and 0.6 per cent. NaCl, and the desmids and *Phacus* did well in Pringsheim's solution.

The hanging drop cultures were, in general, more successful. Culture F, a drop of Sellard's modified liquid, was interesting because of the changes which took place. The flagellates (green variety) went into resting state (Fig. 4) during the first day, but began to resume activity at the end of a week. Some divided in the resting state (Figs. 5 and 6). On resuming activity they first put forth a single flagellum and began to swim about with it, later two more flagella emerged. In some individuals the chlorophyll became unevenly distributed, being concentrated at either end. In others the chlorophyll disappeared entirely, but such colorless individuals retained the form of the green variety. Various species of *Euglena, Phacus,* and desmids multiplied in this culture and *Euglenamorpha* persisted until transplants were made at the end of a month.

In culture K (0.6 per cent. NaCl) there were at the start two or three dozen of the green variety and half dozen *pellucida.* At the end of five days all the colorless individuals were dead, but the type variety had begun to multiply. At the end of three weeks there were between 300 and 400 of the green individuals. The numbers remained stationary for a few days, then gradually declined, but a few survivors were still active after five months. After the first few weeks, many of the flagellates moved to the edge of the drop nearest the window, remained stationary except for the lashing of the flagella for varying lengths of time, and then began to disintegrate. In the disintegrating individuals the paramylon granules often persisted apparently unchanged for days or weeks, while the remainder of the body completely decomposed. Very few entered the resting stage such as shown in Fig. 4.

Culture L was made with the sodium chloride-sodium citrate mixture. During the first five days not more than one of the green variety was visible, but during the following two or three days the number increased to nearly three dozen. When the culture was a week old a binucleate *Opalina* appeared, having probably emerged from a cyst. Multiplication followed till there were sixteen *Opalina* at the end of the five more days. The *Opalina* and *Euglenamorpha* were then transplanted to new hanging drops, but did not survive.

In culture O (Pringsheim's solution) there were between one and two dozen of the green variety at the start. At the end of a week no active individuals could be found, the flagellates having either died or assumed the resting state. At the end of another week several resumed their active condition and remained active with no apparent multiplication for two or three weeks longer. *Euglena* and *Phacus* multiplied readily in this culture.

Culture P was made with equal parts of modified Sellard's liquid and Pringsheim's solutions. During the first three days the numbers increased from about three dozen to over a hundred, but some had already gone into the resting state or begun to disintegrate. Nothing but the green variety could be seen. During the following week the numbers of active individuals gradually decreased and then an individual appeared with the form and activity characteristic of *pellucida*, but a slight amount of color was still discernible. Two weeks later several individuals showed the form and behavior of *pellucida* and in one the four flagella could be made out. Transformation seems to have occurred in this case.

Culture R, like K, was made with a drop of 0.6 per cent. NaCl. Only the green variety appeared at first. There was rapid multiplication for three days followed by a rapid decline of the culture. Some individuals lost all color, but retained the form of the green variety.

Attempts to transplant the organisms to new hanging drops or to dishes were frequently made, but in none of these subcultures did *Euglenamorpha* survive more than a day or two.

Of the total of twenty-six original cultures made, only a few of the hanging drops could be considered successful. These few did demonstrate, however, that the green variety can be cultivated outside the host, that outside the host a resting state may be assumed during which division may occur, and by means of which, presumably, access to new hosts could be obtained, and that the green variety may become colorless and probably change to *pellucida*.

E. Behavior.

One of the striking features of these flagellates is their great activity. No other euglenoid that the writer has ever seen can equal *Euglenamorpha* in rapidity of movement of either the swimming or metabolic ("euglenoid") type. Examined in the rectum of the host, they can be seen swimming rapidly about or stationary in contact with the wall of the rectum or its contents. When teased out into salt solution or water they usually increase their activity, adding the "euglenoid" form changes to the swimming movements, then quickly assume the normal elongated shape, swim actively for a while, then gradually slow down till dead. The pellucid variety with its additional flagella is even more active than the green one, as one might reasonably expect. In cultures where they were able to persist, they often came to rest at the edge of the drop toward the window, the body remaining practically motionless while the flagella continued their activity. In these individuals the flagella usually assumed a position near the body instead of extending out in front. In fresh material examined in salt solution or Ringer's solution, they have been seen to attach themselves by one flagellum while the other two continued to vibrate along the side of the body, and within the rectum a similar behavior has been seen. The activity of the flagella along the sides of the body might have a respiratory function as well as serve to bring food (in solution) to the body for absorption.

As Hegner ('23) has found, *Euglenamorpha* is positively phototropic.

Discussion.

Since Hegner ('23) has found this new flagellate in New York and Maryland and the writer in Pennsylvania and at Woods Hole, Mass., it would seem to be rather widely distributed. It is surprising, therefore, that its existence has not been noted by other observers. One reason is probably the fact that large numbers of green organisms pass through the entire digestive tract of tadpoles without harm. Not only *Euglena* and *Phacus,* but *Trachelomonas, Eudorina,* desmids, filamentous algæ, and such distinctly animal forms as *Difflugia,* rotifers, and entomostracans emerge unharmed from the gut of tadpoles, so that one is inclined to wonder what may constitute the source of nutrition for them. Ordinary species of *Euglena* and *Phacus,* when removed from the rectum and placed in water or even in weak physiological salt solution, will put out a flagellum and swim about. Having seen many cases of this kind, it was with difficulty that the writer first convinced himself of the entozoic habit of *Euglenamorpha.*

Alexeieff ('12) records the finding of numerous *Euglena* and *Phacus* alive and moving in the intestines of tadpoles, and using this fact with the known cases of parasitism among Euglenoids and certain observations on other flagellates and *Sporozoa* supports the theory of the origin of the *Sporozoa* from Euglenoids. In a footnote to this paper he says that Brumpt had shown him preparations from tadpoles in which there were "hundreds and hundreds of Eugleniens." While it is possible for tadpoles to ingest hundreds of *Euglena* at one time, the writer's experience is that *Euglenamorpha* alone occurs in hundreds, so that it is possible that both Alexeieff and Brumpt had observed *Euglenamorpha.*

Haswell ('92, '07) and Beauchamp ('11) report the finding of *Euglena*-like flagellates in the tissues of different rhabdocœl worms, but in each case there was but a single flagellum. Beauchamp's fairly complete description of his *Astasia captiva* reveals a number of points of similarity between it and *Euglenamorpha hegneri* (type). They are similar in size, shape, paramylon granules, and surface striations. They differ, however, in the absence of color and the presence of but one flagellum in *Astasia captiva,* which also has a nuclear structure somewhat different from that of *E. hegneri.*

Since ordinary Euglenoids pass through tadpoles to resume activity upon emerging, the assumption of regular activity within the host would be an easy transition. One naturally wonders if there is a three-flagellated Euglenoid which has not assumed the entozoic habit. None has been found described in the literature, and none has been observed in the water of the pond from which the tadpoles were obtained.

Since Zumstein ('00) and Ternitz ('12) have found that *Euglena gracilis* will lose its chlorophyll and become colorless when supplied with rich nourishment, it is not so surprising that *Euglenamorpha* should lose its chlorophyll and become colorless in the rectum of the tadpoles where it presumably is surrounded by abundant food. Zumstein and Ternitz do not record any morphological changes, other than in the cytoplasmic bodies, accompanying the loss of color in *Euglena gracilis.* It is, therefore, remarkable that *E. hegneri* should exhibit so many structural changes when it loses its color. The differences in shape of body, number of flagella, structure of the nucleus, in surface striæ and in reservoir, in addition to the difference in color, would probably justify the formation of a different genus if the intermediate states had not been discovered. When one realizes that such a transformation does take place it is almost like having seen evolution occur.

Zumstein and Ternitz point out that their results do away with the boundary line between the genera *Euglena* and *Astasia* which had been separated on the basis of the presence or absence of color (chlorophyll and stigma). The differences between the type of *E. hegneri* and its variety *pellucia* are much more extensive and serve to emphasize the indistinctness, at times, of systematic boundaries.

There is nothing in the observations made on the green and colorless variety of *E. hegneri* to indicate that either ingests solid food. Indeed, the writer is extremely doubtful if any of the *Euglenidæ* ever become holozoic. Those bearing chlorophyll must be thought of as largely holophytic in their mode of nutrition, while the colorless ones are probably saprozoic (saprophytic).

Assuming that the green variety of *E. hegneri* transforms into the colorless one, it is perhaps surprising that there are so few intermediate stages. Valid transition stages are, in fact, rather rare. If one were inclined to argue against transformation, the following objections to it could be raised: (1) the great extent of morphological differences between the two varieties; (2) the relative stability of the two forms; (3) the rareness of intermediate stages; (4) the occasional occurrence of large numbers of *pellucida* which may outnumber the type as found on prepared slides; (5) the absence of direct observation of the transformation. It can further be argued that degeneration of the green variety leads to their death, and that the pellucid variety may have a stage in which there is some chlorophyll.

Most of these difficulties can, however, be satisfactorily explained. The colorless variety, while fairly distinct, is too unstable to be a permanent type. This instability is indicated by (1) the probably abnormal hypertrophy and amitotic division of the nucleus; (2) the extreme variation in the surface striations; (3) the variations in the number of flagella; (4) the probably abnormal enlargement of the reservoir (excretory system); (5) its failure, so far as observed, to form a resting stage which could be carried over from one host to another.

The flagellar situation calls for special comment. It is difficult to understand why, by actual count, approximately 40 per cent. of the *pellucida* should have four, 40 per cent. have six, and the remaining 20 per cent. either two, three, or five flagella. Six flagella can be accounted for by assuming an abnormal doubling coördinated with the hypertrophy and amitosis of the nucleus. Three flagella would occur as a result of the division of a six-flagellated individual or as a result of recent transformation from the type. As shown in Fig. 20, transformation may be accompanied by the addition of one flagellum, possibly on account of the hypertrophy of the nucleus, thus giving four. Two-flagellated individuals would result from the division of those with four. The occurrence of five-flagellated individuals is a further indication of instability of flagellar conditions and may indicate the addition of two instead of one or of three flagella.

The rareness of transition stages compared with the number of *pellucida* can be explained as follows: Division of the green variety appears to be rare within the host and when it does occur seems to result in pellucid individuals (Figs. 22 to 26). *Pellucida*, once established, however, seems to multiply relatively rapidly by both mitotic and amitotic divisions, thus enabling it to outnumber the type. The infrequency of transformations would harmonize with the fact that *pellucida* has not been found in any of the tadpoles of *Rana palustris* nor in the tadpoles of other species examined in 1923 up to August 15. In tadpoles examined late in 1922 up to January, 1923, the green variety was still found, but not the colorless one. This fact also points to the green variety as the stable, persistent one, while *pellucida* appears to be neither stable nor persistent.

One is tempted to regard the entire situation here dealt with as revealing an uncompleted series of stages in the evolution of a colorless parasite from a free-living, green euglenoid flagellate. It might be assumed that, since *Euglenamorpha* (type) is still green, it can not have become completely dependent on the tadpoles; that adaptation between the invader and its host is not yet perfected; that in its as yet not thoroughly accustomed surroundings within a host it occasionally undergoes the changes recorded above and becomes the variety *pellucida;* that this variety has not completed its adaptation and has not yet established itself as a regular inhabitant with its own protected stages for reaching a new host. The final steps necessary to establish it as a new species of parasite seem not to be very great, but of this one can not be certain. This is an alluring picture of evolution in progress, yet the information at hand is scarcely sufficient to assert positively that the picture is real.

Summary.

1. *Euglenamorpha hegneri* is the name proposed for a new *Euglena*-like flagellate found in the rectum of tadpoles and first reported by Hegner ('22).

2. Most of the tadpoles examined were those of the bullfrog obtained from the pond in the Botanical Gardens of the University of Pennsylvania. Some tadpoles of *Rana palustris* collected by Dr. C. L. Parmenter and fed on vegetation from the same pond were also examined. About 60 per cent. of the 117 bullfrog tadpoles examined in 1922 and 16 per cent. of the 30 examined in 1923 harbored *Euglenamorpha*. About 60 per cent. of the 9 tadpoles of *Rana palustris* examined in 1922 and also of the 35 examined in 1923 were infested with this flagellate. Eight tadpoles of *R. clamitans* (?) and 8 of *Hyla versicolor* (?) examined at Woods Hole in August, 1923, all contained *Euglenamorpha*.

3. There are two varieties: one, green, is the type, and the other, *pellucida*, is a colorless derivative. Typical individuals of the two varieties are so different that without intermediate stages they would be put into different species or into different genera.

4. The green (type) variety has a cylindrical or cigar-shaped body, chlorophyll, stigma, paramylon granules, compact nucleus, three flagella with swellings on their roots near the stigma, surface striæ passing from the left over to the right. It may occasionally divide by mitosis within the host.

5. Variety *pellucida* has an elongately conical body, little or no chlorophyll, no stigma, no swellings on the usually four or six flagella; has enlarged reservoir, nucleus expanded, and surface striæ usually passing from right over to the left or longitudinal. Amitosis as well as mitosis occurs frequently in the host.

6. Transitions between the two varieties are found and it is believed that the green variety transforms into the colorless one.

Division of the green variety in the host is rare, but appears to be accompanied by transformation which may also take place without division. Once established, the colorless variety may multiply rapidly by both mitosis and amitosis and consequently outnumber the green one.

7. Attempts to culture the flagellates outside the host were more successful with hanging drops than with dishes. The most successful culture was a hanging drop of 0.6 per cent. NaCl in which the green variety multiplied for three weeks, then gradually declined, but some few were still alive after five months. In other cultures a few appeared to transform from the green to the colorless variety.

8. The movements of *E. hegneri,* both swimming and metabolic, may be very rapid. In some cases the organisms appear to attach themselves to the wall of the rectum or other object by one flagellum and to vibrate the others near the body, thus possibly serving both a respiratory and a nutritive function. They are positively phototropic.

9. Outside the host (in culture) the green variety may assume a resting state approximating encystment in which it may divide and by means of which, presumably, it reaches a new host. The colorless variety is not known to assume the resting state and appears to be too unstable to be a permanent form.

10. One is tempted to regard the entire situation as revealing an uncompleted series of stages in the evolution of a colorless parasite from a free-living, green euglenoid flagellate, but the evidence is not entirely conclusive.

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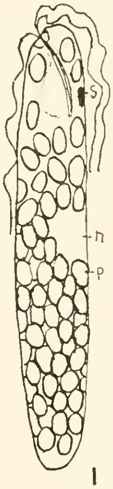
EXPLANATION OF FIGURES.

Plate I.

Figs. 1–6. From living material. × 1,100. Fig. 1, normal shape of green variety showing paramylon granules, *p;* stigma, *s;* position of nucleus at *n*. Figs. 2 and 3 show metabolic form changes. Figs. 4–6, resting state; division in Figs. 5 and 6.



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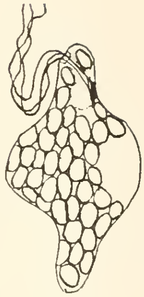

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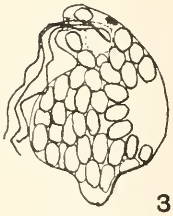

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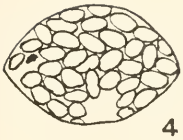

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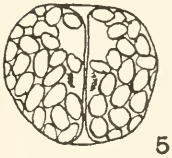

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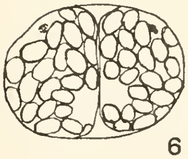

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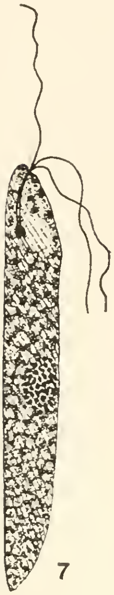
Plate II.

Figs. 7–16. From prepared slides. × 1,100. Figs. 7–11, type variety; Fig. 7, usual appearance; Fig. 8, surface striæ longitudinal; Fig. 9, striæ passing from right to left; Figs. 10 and 11, show loss of chlorophyll and expansion of nucleus; Figs. 12–16, variety *pellucida*. Note four (Fig. 12), five (Fig. 14), and six (Figs. 13, 15, 16) flagella, and caryosomes from one (Fig. 12) to four (Fig. 16).



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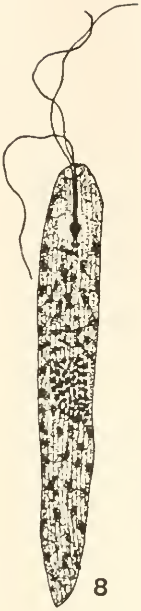

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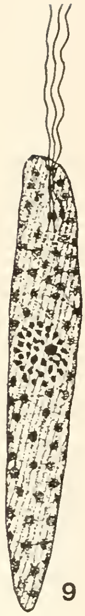

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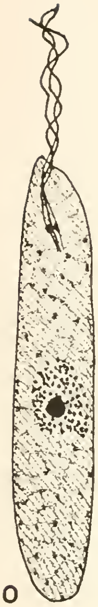

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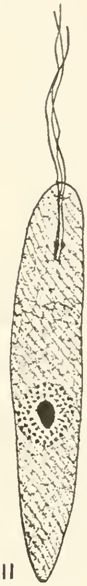

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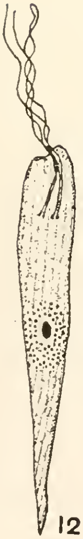

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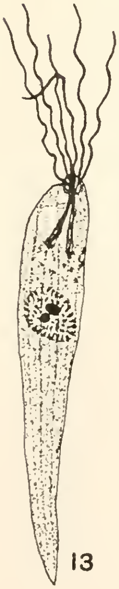

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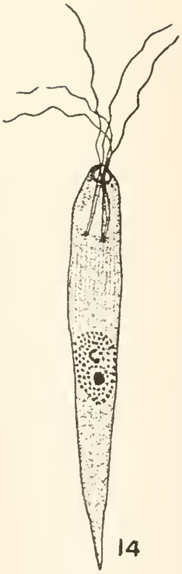

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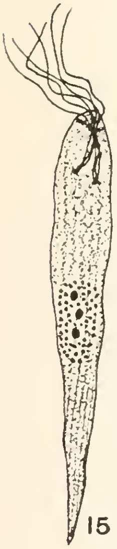

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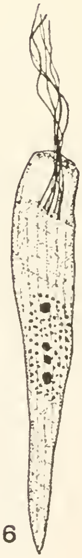

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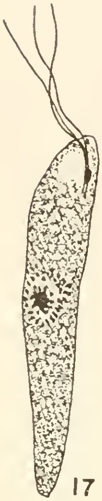
Plate III.

Figs. 17–26. From prepared slides. × 1,100. Figs. 17–21, transitions between varieties; Fig. 17, chlorophyll breaking up, nucleus expanding; Fig. 18, chromatic swellings nearly gone from flagella, chlorophyll partly gone; Fig. 19, some chlorophyll present, striæ changing position; Fig. 20, one extra flagellar root, chlorophyll mostly gone and nucleus expanded; Fig. 21, pellucid characters except for presence of chlorophyll; Figs. 22–25, stages in mitosis of type variety accompanied by gradual loss of swellings on roots of flagella; Fig. 26, probably a daughter cell.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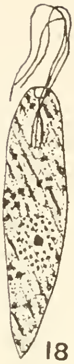
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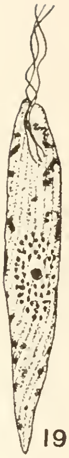
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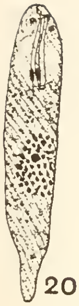
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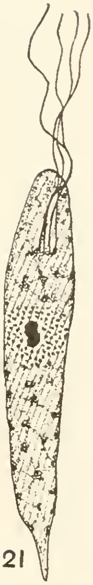
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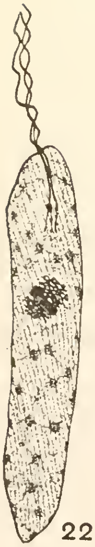
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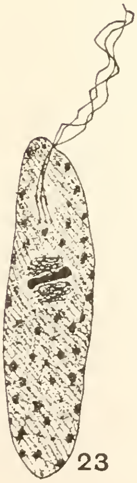
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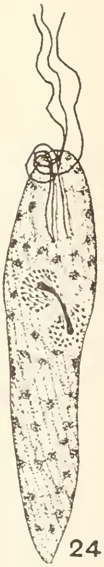
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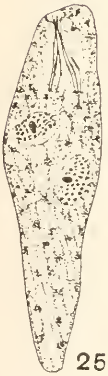
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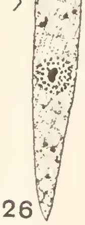
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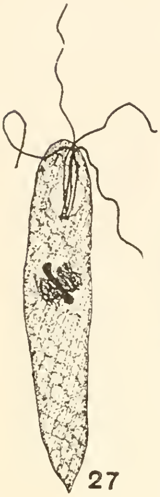
Plate IV.

Figs. 27–36. From prepared slides. × 1,100. Figs. 27–31, stages in mitosis of nucleus of *pellucida*. Figs. 32–35, stages in amitosis of nucleus of *pellucida*. Fig. 36, daughter cell with only two flagella.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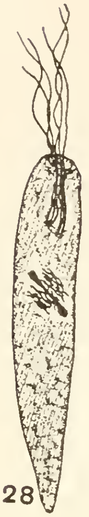
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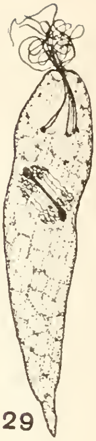
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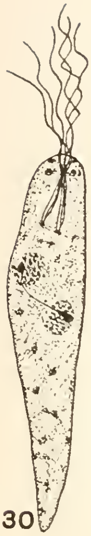
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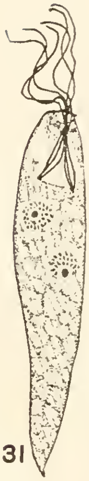
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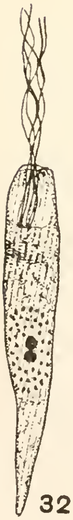
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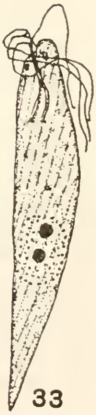
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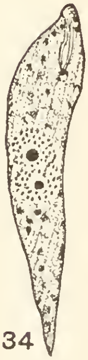
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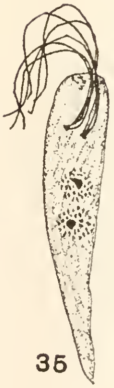
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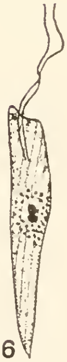
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D. H. WENRICH.

SEX-RATIOS AND SPERMATOGENESIS IN THE TOP-MINNOW, *GAMBUSIA HOLBROOKI* GRD.

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WASHINGTON UNIVERSITY, SAINT LOUIS.

(From the Zoölogical Laboratory of the Johns Hopkins University.)

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I. INTRODUCTION.

Sex-Ratios Reported in Gambusia and other Pœciliids.—Many observations have been recorded of the sex-ratios in field collections of *Gambusia* and other viviparous members of the family

TABLE I.

REPORTED SEX-RATIOS FOR PŒCILIID FISHES.

Species.	Locality.	Ratio x ♀ : 100 ♂	No. of Indiv.	Remarks.	Authority.
Cnesterodon 10-maculatus	Central and South America	150	5		Henn, 1916
Diphyacantha chocoensis	" " " "	357	32		" "
Fitzroya lineata	" " " "	600	7		" "
" *maculata*	" " " "	533	23		" "
Gambusia affinis (= *holbrooki*)	Lower Potomac River	High!	69	"1 male to 68 females"	Smith, 1892
" " "	" " "	"	95+	"Several males and abt. 90 females"	Smith, 1912
" " "	Pasquotank R., N. C.	300	—	"One out of four males"	Smith, 1893
" " "	North Carolina	High!		"Males only 2%–3% of a population"	Smith, 1907
" " "		High!		"Males very rare and not often collected"	Jord. & Ev., 1896
" " "		800–900			Hildebrand, 1917
" " (= *affinis*)	Mound, La.	187–882	Many!		Barn. & Ans., 1921
" " (= *holbrooki*)	Beaufort, N. C.	257	693		Geiser, 1921
		775	796		" "
" *punctata*	W. Cuba	High!			Eigenmann, 1904
" *puncticulata*	W. Cuba	High!			" "
Glaridicthys uninotatus	W. Cuba	High!			" "
Jenynsia lineata	*	500			Weyenbergh, 1863
" "	*	Low!!		"Freq. purely ♀ litters one after another"	Thumm, 1908
Lebistes reticulatus	*	100–250		Diff. survival rates at diff. temps. With diff. sex-ratios of litters	Boulenger, 1912
" "	Barbadoes	100		"Normally a 1 : 1 ratio"	Henn, 1916
" "	*	116	Many!!		Schmidt, 1920

TABLE I (continued).

Species.	Locality.	Ratio x♀ : 100♂.	No. of Indiv.	Remarks.	Authority.
Leptorhaphis infans	Mexico	High!	78		Meek, 1902
Limia vittata	W. Cuba	156		"Fewer males than females"	Eigenmann, 1904
		200	82		" "
Limia hollandi	Central and South America	1333	105		Henn, 1916
Mollienisia formosa	*	High!!!		"Purely fem. litters one after another"	Thumm, 1908
Mollienisia latipinna		78	71		Everm. & Goldsb, 1902
			16		
" *occidentalis*	Rio Yaqui, Sonora	267			Rutter, 1896
" *spilurus*		High!!!	33		Stansch, 1911
" "		High!!		"Progeny almost all females" "2 or 3 ♂ to 40 or 50 females"	Milewski, 1920
Neoheterandria elegans	Colombia	300			Henn, 1916
Phalloceros caudomaculatus	S. Amer.	334	16		Henn, 1916
Phalloptychus eigenmanni	Bahia, S. Amer.	450	262		Henn, 1916
Platypoecilus maculatus	*	148	11		Bellamy, 1922
Poecilia parae	British Guiana	136	669		Eigenmann, 1909
		362	38		
" *vivipara*	Brazil	656	37		Henn, 1916
Pœciliopsis presidionis	Colombia	400	121		Eigenmann, 1912b
Priapichthys episcopi	Canal Zone	16	5		Everm. & Goldsb. 1909 and 1910
	" "	900	29		
" *nigroventralis*	Colombia	110	10		Eigenmann, 1912b
	Central and South America	127	21		Henn, 1916
Pseudoxiphophorus Sp.	Mexico	High!	34		Jord. & Everm., 1896
" *bimaculatus*	*	Low!!		"Nearly all ind. in 2 litt. males"	Langer, 1913
Tomeurus gracilis	British Guiana	100	6		Eigenmann, 1909
Xiphophorus helleri	" "	68	317		Bellamy, 1922

* Aquarium populations.

of the top-minnows or *Pœciliidæ*. The results in reference to sex obtained in these collections are presented in Table I. By referring to this Table it will be seen that in practically all ratios reported for *Gambusia*, and in 80 per cent. of the records of the pœciliids generally, the females were greatly in excess.

It has been suggested that the discrepancy in the proportions of the sexes is due to the fact that in this group of fishes the males are much smaller and more agile than the females, and hence either more readily avoid, or pass through the meshes of, the net. However, in those cases where the net used had a mesh so small that loss of the smaller males was thereby excluded, collections still showed the same inequalities of numbers of the sexes. It therefore seems well-established that in the field there is usually, if not always, a marked excess of females in these fishes. The writer has had populations under observation in which the proportion of males present was as low as 3.8 per cent. By the method employed in catching them it was not possible for a single individual, male or female, to escape.

Moreover, Hildebrand ('17) observed the sex-ratios of specimens of *Gambusia* raised in the laboratory under conditions in which every individual was examined, and found them to be approximately the same as those observed in collections taken in the field. He concludes ('17, p. 10) that "It seems entirely probable that the normal ratio of males to females is about 1 to 8 or 9."

Mast ('21) found on dissection that, in some populations of Gambusia that he studied, a considerable proportion of the individuals which looked like females and had been counted as such were really males in which the secondary sex-characters were not developed. He designated these "sterile males" and suggested that the reports of excessive numbers of females might be due to the fact that many such males might be counted as females. However, in the course of the writer's studies, when large numbers of "females" from populations showing low percentages of males were dissected to ascertain whether a considerable number of these "females" might not really be sterile males it was found that the percentages of sterile males in such populations was too small to account, even in a small degree, for the very great disproportion in the numbers of the sexes.[1]

It must therefore be concluded that the inequality of the sexes of adult Gambusia is real, and cannot be accounted for on the basis of faulty methods of collection or of inability to recognize the sexes. This fact has led some investigators to hold that sex in Gambusia is conditioned by obscure fluctuating environmental or physiological factors, and that it is not unalterably determined at the time of fertilization. Conclusions similar to this have been held by Woltereck ('08) in reference to various Pœciliids, and by Okkelberg ('21) in reference to the brook-lamprey.

Sex-Ratios in Fishes.—But little work has been done on the sex-ratios of fishes generally, and nearly all of this only on populations not under experimental conditions. In this work the enumeration was only of adults, usually by superficial examination only, and hence the value of the findings is very uncertain in the presence of so many variables. That is, the sex-ratios reported for fishes have in nearly all cases been simply "sex ratios of collection," and explicable upon a number of hypotheses. Darwin ('75) and Fulton in various papers, have largely given us what little data we have on the subject.

Among the factors that might cause atypical ratios in adult fish two alternative ones occur most readily to the mind: (*a*) a possible differential death-rate of the sexes during the embryonic, juvenile, and adult period, coupled with a normal sex-ratio at fertilization, and (*b*) an atypical primary sex-ratio, due to an atypical distribution of sex-determining chromosomes to the two daughter cells in the maturation divisions of the germ-cells. As a result of this unequal distribution of the sex-chromosomes, a preponderance of one sex over another could conceivably be produced. The approximate proportions found

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[1] Essenberg (BIOL. BULL., Vol. 45, pp. 46–97, 1923), in his very interesting paper finds that in old populations of *Xiphophorus* the percentage of males is often very high. He concludes on the basis of his observations that in this species there may be large numbers of "retrogressive females" which later transform into males. In my observations on *Gambusia* I have never found intersexes or instances of sex-inversion.

(one male to three or seven females) are suggestive of this explanation of the atypical adult ratio.

The special chromosomes whose presence or absence appears in many animals to be associated with a definite sex have not yet been reported[2] on cytological grounds for any teleost fish. If we knew that such a chromosome (or chromosomes) existed in the sex-cells of a given species, thus giving rise to dimorphic zygotes on fertilization, we could forecast approximately equal numbers of the sexes at birth (secondary sex-ratio of Schultz, '18), unless the gametes showed a differential viability. This cytological method of ascertaining primary and secondary sex-ratios is of course the most dependable one we have. Failing in this, valid conclusions may also be reached by taking large litters and raising them with as few mortalities as possible to a stage where the sexes may be distinguished. Unless the early sex-ratios are ascertained by one of these methods, the results are quite untrustworthy. Apparently only three workers, Eigenmann ('96), Punnett ('04), and Hubbs ('21) have done this. The first mentioned, in his work on the viviparous Embiotocid *Cymatogaster* ascertained the sex-ratio of litters before birth by microscopical examination of the embryo-gonads, and found the sexes approximately equal in numbers in the litters. Punnett ('04) in a similar way found the sex-ratio in the Elasmobranch *Spinax* to be approximately a 1 : 1 ratio, and Hubbs ('21) obtained results similar to those of Eigenmann in the Embiotocid teleost, *Amphigonopterus*. Only the sex-ratios of adults can be learned by external observation of the individuals of a population. It is certain, of course, that if no differential death-rate of the sexes exists in a given species, the sex-ratios of adults will give a trustworthy index to the sex-ratios at fertilization and at birth, as appears to be the case in the lake whitefish, *Coregonus albus*, as reported by Pearl ('16).

As has been said, in *Gambusia* the females are greatly in excess of the males. The following cytological and experimental studies were undertaken with the view of ascertaining whether this is owing to peculiarities in the distribution of possible sex-determining chromosomes, or to a differential viability in the gametes, or the zygotes, both in embryonic and juvenile stages.

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[2] No differences have been found between the sex chromosomes and the autosomes in the poeciliid teleost, *Lebistes*. (Winge, '22*a*, also in C. R. Lab. Carlsberg, Vol. 14, No. 17, p. 8, 1922.)

II. Spermatogenesis in *Gambusia,* with Special Reference to its Bearing on the Explanation of the Sex-Ratio.

The illuminating genetic studies of Schmidt ('20) have demonstrated that *Lebistes* has an XX, XY constitution. Aida ('21) has also shown the same thing to be true of another Pœciliid fish, *Aplocheilus.* Since *Gambusia* and *Lebistes* are closely related it is reasonable to assume that the chromosomal arrangement in these two forms is similar. If this is true, then we should expect in *Gambusia* equal numbers of potential males and females at the time of fertilization, and if this is what actually obtains, then the preponderance of females observed in adult populations must be due to a differential death-rate operating after fertilization. The importance of the investigation of spermatogenesis in this form is consequently obvious.

But little work has been done on the spermatogenesis of teleost fishes. The only title that the writer has been able to find is that by Turner ('19) on the seasonal cycle of the testis of the perch.[3] Bohm ('91) and Behrens ('93) have investigated the oögenesis of the "Forrelle" and Blanc ('94) has studied the oögenesis of *Trutta lacustris.* Mrs. Harvey ('20) in her synoptic paper has noted brief observations by other authors on diploid cleavage numbers in a number of other teleost fishes. But except for the paper of Turner above noted no study appears to have been made of the male sex-cells of teleosts. The explanation seems to lie in the fact that in this group of fishes the chromosomes are very numerous, small in size, and lack individuality.[4]

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[3] Since the above was written, Winge ('22*a*) has briefly described the spermatogenesis and oogenesis of *Lebistes reticulatus,* and Essenberg ('23) has published brief observations on spermatogenesis in *Xiphophorus,* a poeciliid fish.

[4] In *Lebistes* and *Xiphophorus* the same difficulty is found. (*Cf.* Winge. '22*a,* and Essenberg, '23.)

Materials.—The *Gambusia* used in this study were from the P_1-stock of the experimental litters (*q. v.* in Section III. of this paper). Thus the material for both cytological and breeding studies was from the same source. The stock had a very great preponderance of females, the males being outnumbered by the females nearly eight to one.

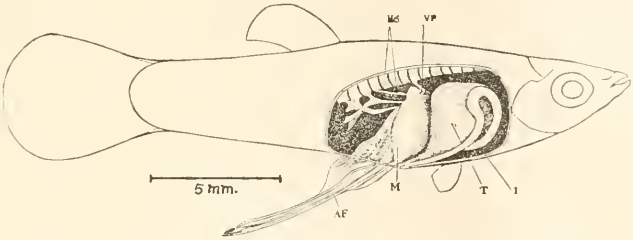
Methods.—Preliminary tests with Flemming's solution, and with many modifications of Bouin's fluid, particularly Allen's ('18) modification, demonstrated the fact that the best fixation of prophase stages in the material was obtained by the use of Bouin's fluid, modified by the addition of one per cent. of Merck's *c. p.* urea crystals. This was the fixing agent employed in all subsequent work on the cytology of Gambusia. With the best of care, however, it was not possible with any fixing solution to get good separation of the chromosomes in the spermatocyte metaphase plates, even though the earlier phases in the same section were beautifully fixed. This difficulty in working with teleost testes is one that has troubled a number of workers. Turner ('19) in his work on the perch, e.g., was unable to obtain spread equatorial plates of chromosomes.

Both section and smear preparations were made. Sections were cut five and seven micra thick. All preparations were stained by Dodd's ('10) long process, with iron-hematoxylin. Occasionally counter-stains were used.

The General Anatomy of the Testis.—In the adult fish the two testes are fused, forming a single gland lying just below the posterior portion of the swim-bladder, and anterior to the gonopod (Text-fig. 1). The posterior portion of the intestine lies below and anteriorally to the combined testes (hereafter called the "testis"). The large muscle controlling the movement of the gonopod lies just behind the testis.

The testis is whitish in color. It lacks the heavily pigmented investing membrane so characteristic of the ovary. It is suspended from the swim-bladder and the vertebral column by a very thin mesorchium. The surface of the testis when examined in a fresh condition shows a more or less favose network of cell-borders under the thin investing membrane. These nettings are caused by the arrangement of the cysts in the testis.

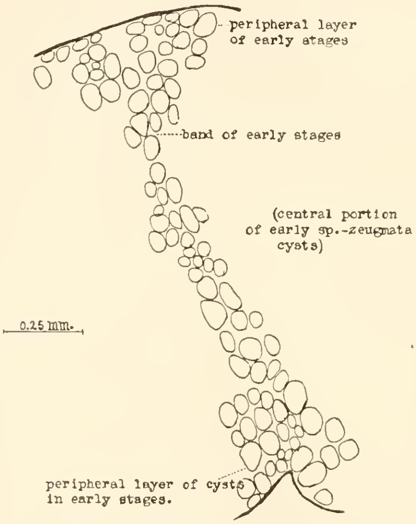
The testes vary in size with the season and the individual. Thus, the median length of 19 taken at random on July 20, 1920 was 3.2 mm. and the median breadth 2.2 mm.; while the median length of five taken November 23, 1920 was 2.75 mm. and the median breadth 2.07 mm., a volume relation of approximately 1.6 to 1. The testes in individuals of the same litters vary but little in size, but they vary greatly in individuals of the same size taken from different litters.



Text-Figure 1. Lateral dissection of a male *Gambusia holbrooki* showing the general relations of gonopod to other organs, especially the internal organs of the body. *I,* intestine; *T,* testis; *AF,* gonopod; *HS,* modified haemal spines; *M,* muscle controlling gonopod; *VP,* ventral process of abdominal vertebrae. (After Kuntz, '14.)

There is no prominent connective-tissue core in the testis of Gambusia such as Turner ('19) described for the perch, and Brock ('78, Taf. xxviii, Fig. 3) figured for *Perca fluviatilis.* The testes of *Gambusia* consist of a tenuous, diffuse, connective tissue stroma, in the interstices of which the spermatogonia and cysts are closely packed. The structure of the adult testis resembles very closely in some respects that observed by von Ihering ('83, p. 486) in the South American pœciliid, *Phalloceros caudomaculatus.* The smaller cysts of multiplying spermatogonia lie in the cortical portion of the testis. As one proceeds inward the cysts become more and more mature. The cysts in the spermatid stage are much larger than the mature sperm-balls, or spermatozeugmata. The latter lie closest to the center of the testis, and near to (and with) the longitudinal canal, which appears to serve as a storage place for sperm; the former, more peripherad. After the growth-period preceding the I.-spermatocyte division, the cysts are much larger than before. von Ihering's description of the formation of a spermatozeugma in *Phalloceros* holds also for *Gambusia.*

The testis of *Gambusia* lacks the tubular type of structure characteristic of the testes of oviparous Pœciliids, *e.g. Fundulus.* Apparently there has come with viviparity in this family fertilization of the eggs of the female by means of sperm-balls, which appear to be formed in the same way in all viviparous members of the family *Pœciliidæ* examined.


peripheral layer of early stages
band of early stages
(central portion of early sp.-zeugmata cysts)
0.25 mm.
peripheral layer of cysts in early stages.

TEXT-FIGURE 2. A portion of the testis of *Gambusia,* showing the differential development of spermatocytes indicative of the original paired condition of the testis.

In the adults the two testes are very closely fused; the double structure being evidenced only by the almost universal separation of the longitudinal testicular canal into two portions, one ramus on either side of the median line in the anterior four-fifths of the testis; and in the differential development of spermatocytes (Text-fig. 2).

In the testis figured the major portion of the gonad consisted of cysts in spermatid and early spermatozeugma-stages. These occupied the central portion of the testis, grouped about the paired longitudinal testicular canals. At the periphery there were cysts whose cells were in early I.-spermatocyte division prophases. Along the median plane there also occurred a band of cysts whose cells were in the same stage of mitotic division as those of the cysts occurring at the periphery of the testis. The line of fusion of the two original testes appears clearly in the vertical band of younger cysts running through the section figured.[5]

Seasonal Variation in the Adult Testis.—The testis shows a marked seasonal periodicity in volume, the number of contained spermatozeugmata, and the volume of the longitudinal testicular canal. During the spring and summer, which is the breeding season, the testis increases in size. The increase is, however, not large, as it averages only 60 per cent., while the maximum is only a sixfold volume-increase. This is much less than has been found for other fishes, e.g., the perch.

During the late autumn and winter and very early spring the testis is filled with spermatozeugmata which greatly distend the longitudinal testicular canal. After the great spring wave of copulation the testes show very few spermatozeugmata, and in June and July the testes are filled with early stages of spermatogenesis. Smears of the testes taken in August and September show only spermatids and spermatozeugmata along with late spermiogenesis stages.

The Spermatozeugmata.—When ripe, the spermatozeugmata are spheroid to ovoid bodies with a diameter in winter testes of 210-320 micra. They lack completely the investing membrane characteristic of spermatophores and agree entirely in structure with those described for *Cnesterodon 10-maculatus* by Philippi ('08.) It is probable that the error into which Kuntz ('14) has fallen with regard to the structure of the "spermatophore" is due to his having studied and described cysts containing immature spermatozoa. For in mature cysts and spermatozeugmata the spermatozoa lie at the periphery and have a definite arrangement.

Observations on Spermatogenesis. (*a*). *Primary and Secondary Spermatogonia.*—The definitive sex-cells by repeated divisions give rise to the final spermatogonia. It is not known how many fissions take place before the spermatogonia are produced which give rise to the final spermatogonial cyst. A computation based on the approximate total number of cysts produced during the lifetime of a male Gambusia gave the number of fissions as at least 16. Such a computation is to be accepted with reservations: certain it is, however, that there are many cell-divisions from the time of the first segregation in the Keimbahn to the time when the cyst-forming spermatogonia are finally located in the testis.

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[5] Essenberg's "Figure 38" also shows very well the similar bilaterality of the testis in the related species, *Xiphophorus helleri.*

The time of segregation of the first sex-cells is not known. My earliest *Gambusia* embryos have a snout-anus length of 1.58-2.05 mm. Definitive sex-cells are then present in the typical location beneath the swim-bladder. At birth the gonads are developed but slightly beyond the condition found in these embryos. The sexes are not readily distinguishable. The gonads at birth have large, clear cells with a very clear nucleoplasm in which lies a prominent plasmosome. From the plasmosome, and apparently taking their origin from it radiate delicate linin fibrils, upon which frequent granules of deeply staining chromatic material may be seen. The filaments appear to extend to the inner surface of the nuclear wall. Occasionally the sex-cells in the gonad appear especially large and clear, and each cell is invested with a layer of mesoderm cells, but these have been observed in only a few individuals. It is not improbable that such cells are developing oocytes, but the evidence in the present case is not completely convincing. In a few of the individuals cells unmistakeably developing ova are found at birth. These cases are however, rare. In those cells which have not yet differentiated there is usually a prominent plasmosome, with one or more other conspicuous irregular masses of chromatic material. In the later spermatogonia (Figs. 1-2)[6] which are produced from localized peripheral germ-cells of the testis, in the cyst during its formation, there are usually two prominent karyosomes. The final spermatogonia (Figs. 8-10) are approximately one-eighth the volume of the cell which by dividing formed the cyst. These final spermatogonia possess a very fine reticulum of inconspicuous fibrils; the chromatin flakes are small and seen only with difficulty.

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[6] "Figures" refer to the plates.

The early Spermatogonial chromosomes in multiplication-divisions in the cyst (Figs. 3-5) are simple rod-shaped elements, with apparently terminal spindle-fiber attachment. In no case was a J-, U-, or V-shaped chromosome observed in a polar view of the plates. The chromosomes in the spermatogonial metaphase plates are usually so numerous and so densely massed that a dependable count is almost precluded. The number, however, is not less than thirty nor more than thirty-six; in a single widely-spread plate 36 chromosomes were clearly seen. That this is the diploid number is suggested by the fact that in the diakinesis stage preceding the first spermatocyte division, counts of the tetrads give 15 and 18 as the number of quadrivalents. The diploid number of chromosomes in the male germ-cells would then be 36 if no heterochromosome were present, or 35 if one heterochromosome were present. On the latter point it will not be possible to speak definitely, since the II.-Spermatocyte plates are always too crowded and dense to show the exact number of chromosomes. It is apparent, however, that approximately 18 univalent chromosomes are present. Lagging chromosomes are frequently seen in anaphase stages viewed from the side, but not constantly enough to give a clue as to the number, size and form of possible heterochromosomes. Occasionally precocious chromosomes appear in early maturation-stages. If one were to trust the uncertain help of analogy, however, it might be claimed that the male *Gambusia* has one *heterochromosome*. (*Cf*. Schmidt, '20.) With this assumption, it would be quite within the bounds of probability that the spermatogonial chromosome number is really 35, the plate which showed 36 merely possessing a lagging chromosome belonging to the other daughter cell.

By repeated mitotic divisions the cysts become filled with the final spermatogonia (Figs. 8–10). The precise number of fissions of the spermatogonium forming a cyst is not known: computations indicate that at least 10, and often 12 fissions occur before the cysts are ready for spermatogenesis. In a given cyst the cells are all in the same stage of maturation and thus, as in insect testes, it is possible to find all stages of the process, each in a different phase, in a single section.

(*b*) *Later Stages in Maturation. Leptotene. Synapsis. Pachytene.*—In the further maturation of the male germ-cells of Gambusia the process follows the usual path. There are no features worthy of special note. The filamentous chromosomes of the leptotene (Figs. 11-16) are produced by a condensation of the chromatin on the linin fibrils. There has not been any striking evidence of pairing of the chromosomes in the leptotene, owing largely to the difficulty of the material. In the synaptene and pachytene stages (Figs. 17-18) which follow upon the leptotene there is evidence that parasynapsis occurs. The chromatin threads in early synapsis become only half as numerous as in the leptotene. The pachytene shows the typical orientation of the thickened and shortened threads of this stage. No contraction-stages (*synizesis*) appear to occur in this species.

(*c*) *Tetrads and Diakinesis.*—The tetrads in their early stages appear to be of the open-ring type (Figs. 20–21). They contract however, as they take on the peripheral position characteristic of diakinesis (Fig. 22) until all trace of their tetrad character is lost. They then appear as large, spherical, deeply-stained bodies evenly distributed through the plasm just beneath the nuclear surface. In this position they remain for some little time, after which they gather to form an equatorial plate (Fig. 23).

The tetrads are halved in the I.-Spermatocyte metaphase. The distribution of the dyads to the two daughter cells of the first spermatocyte division could not be made out (Figs. 24–26). The II.-Spermatocyte division follows immediately upon the conclusion of the first division, with no nuclear reconstitution. After the secondary spermatocyte division is completed the chromosomes clump together, the chromatin changes in reaction to stains and becomes scattered throughout the nucleus. After the resultant spermatid nucleus has been constituted, a rather long interkinesis occurs. This is followed by a stage in which the chromatin gathers at the periphery of the nucleus and stains deeply.

(*d*) *Spermatozoa.*—The spermatids lose their cytoplasm; the nucleus becomes hemispherical (Fig. 31) then elongate (Fig. 32-33). As the spermatozoa come to nearly attain their mature form (Fig. 34) they migrate to the periphery of the cyst. The heads are directed outwardly, and the tails, together with a colloidal substance, fill the central cavity, precisely as shown by Philippi ('07) for *Phallocros caudomaculatus.* These spermatozeugmata appear to retain their individuality until their discharge from the genital pore of the male. The first spermatozoa are formed in May and June, and the process continues until October. Their formation, then, is contemporaneous with the period of warm weather.

(*e*) *Dimorphism of the Spermatozoa.*—Zeleny & Faust in 1912 made the discovery that in certain insects, the males of which are heterozygotic, the ripe spermatozoa can be divided into two groups which differ considerably from each other in size, those in each group varying about a mode with but very little overlapping. Later work by these investigators (Zeleny & Faust, '15*a*, '15*b*) extended their early results to several groups of insects, and Wodsedalek ('13, '20) found the same dimorphism in the spermatozoa of mammals. In *Gambusia,* the writer was not able to find any such dimorphism. It is possible that when spermatozoa from different spermatozeugmata are measured. and the measurements grouped together, as was done in the present case, a complicating factor enters in the fact that the sperm-heads may thus be in different stages of maturity, and hence of diverse volumes. In view of the facts, however, that the sex-chromosome, if it occurs, is not very different in size from the other chromosomes of the cell, and that the chromosome number is so large, thus making the relative increase in size of the gamete possessing the sex-chromosome slight, it is doubtful if such measurements of sperm heads would furnish any clue to the existence of a sex-chromosome. Whether such a chromosome exists or not may be much more easily ascertained by breeding experiments involving sex-linked characters, as e.g., Schmidt's work, already referred to.

From the results obtained by the study of the spermatogenesis of *Gambusia*, no light is cast on the origin of the anomalous sex-ratio of adults. The cytological evidence, while not satisfactory, points to an apparently nearly equivalent division of the chromosomes. The presence of heterochromosomes is indicated. The cytological condition of related forms would seem to indicate the production of equal numbers of male and female-determining gametes. What the proportions of the sexes in experimental litters with known life-conditions would be, remains to be ascertained.

(*f*) *Summary on Spermatogenesis.*—The testis of adult *Gambusia* is formed by the median fusion of two original paired testes. This fusion takes place within the first two months of independent life. Traces of the double character of the adult testis are shown in the usual division of the longitudinal testicular canal, and in differential maturation along the median plane (line of fusion). The testes in summer are somewhat larger than in winter. The relative proportions vary markedly with diverse individuals. The fluctuation in volume is not so great as in other teleosts.

The testis lacks any strongly-developed connective tissue core. There are no spermatic tubules. The cysts of spermatozoa are the result of the continued fission of germ-cells which at the season of sexual activity migrate to the periphery of the gonad.

The gonads at birth are undifferentiated, but sex is ascertainable within four weeks after birth, food and other conditions being optimum. The period of active sexual life is the spring and summer months. Active spermatogenesis occurs only at this time.

There are no special features of spermatogenesis in Gambusia. The cysts are produced by 10-12 fissions of an original spermatogoium. The final spermatogonia pass through the usual prophase-stages. Each cyst contains approximately 1500-3250 final spermatogonia, all in the same stage of development. During spermatogenesis, this relation continues.

It appears from counts of spermatogonial metaphase plates and from diakinesis stages that the diploid number of chromosomes in *Gambusia holbrooki* is 35 or 36. There is no evidence in the spermatogenetic process of an unusual method of chromatin distribution that would explain the occurrence of the atypical sex-ratio observed in the adults.

III. OBSERVATIONS ON SEX IN *Gambusia* RAISED IN AQUARIA WITH SPECIAL REFERENCE TO ITS BEARING ON THE PROBLEM OF SEX-RATIOS.

Observations on the Sex-Ratios of the Aquarium Populations.—During the years 1919-1920 a series of experiments with gravid *Gambusia* was conducted with a view to ascertaining whether it were possible in these fish to alter the sex-ratios of the young at birth by the feeding of male gonads to the pregnant females. It was known that the males and females cannot be distinguished by superficial observation, but it was assumed that they could be distinguished by cytological methods. Consequently the young of the experimental females were taken at birth, or several days later, fixed in Bouin's fluid, and the entire animal later sectioned and studied. It was found on studying the sections thus obtained that while in some of the individuals unmistakeably definitive oocytes occurred in the usual position just beneath the air-bladder, in others the gonads were still undifferentiated. No internal structures, such as genital ducts or other features gave an indication of the sex. It was consequently evident that the sex of Gambusia at birth cannot be ascertained by cytological methods.[7]

The next endeavor was to raise the young in laboratory aquaria until the sexes were distinguishable. At birth they cannot be so distinguished. In adults it can be done by superficial examination of the anal fin, or by dissection. At the age of three weeks after birth it can be done by the cytological method, and at the age of three months, if life-conditions are optimum, by the examination of the anal fin.

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[7] In this connection, it is interesting to note that since the above was written, Champy & Gley (*Arch. d'Anat. microscopique,* T. 19, Fasc. 2, p. 259, 1923) have found with the poeciliids *Haplochilus, Poecilia, Lebistes, Xiphophorus,* and *Platypœcilus* a very tardy development of the ovary of the young fish. They note that the state of maturity of the gonads is not attained until the animal has acquired its adult size, which is here sharply defined, and that the gland remains in an immature state in animals almost as large as the average-sized sexually mature fish.

These experiments endeavoring to raise the young in laboratory aquaria until the sexes were distinguishable came to naught, for the mortality in indoor aquaria was so great that even with the best of care and feeding the differentiation of the sex-glands did not occur during the maximum period that it was possible to keep any considerable portion of the litters alive. Microscopic study of those individuals which had died during the experiments cast no light on the proportions of the sexes of these fish.

Before proceeding further it may be well to consider the criteria of sex in young and adult Gambusia.

Criteria of Sex in Young and Adult Gambusia.—The viviparous Pœciliidæ, such as *Gambusia*, possess a well-marked sexual dimorphism. The males are much shorter and more slender than the females. The anal fin in the male is placed farther forward than in the female, and is modified into a conspicuous "intromittent" or copulatory organ, the *gonopod*. This dimorphism of the sexes is so marked that in one species male and female were originally classified in different genera, and their identity was disclosed only after a lapse of years, when individuals of the assumed two genera were observed to copulate. The sexual dimorphism of the adult constitutes a very serviceable criterion of sex, but in juvenile, immature forms it is inadequate. Studies to ascertain the limits of variation of sex-characteristics, such as position of the anal fin in male and female, were therefore made by the writer in order to gain data which would permit one to ascertain readily, without dissection, the sex of an individual suspected of being a "sterile male." A study was also made of the development of the anal fin in the male from the indifferent stage to that of a fully-developed gonopod, for data concerning the time of first appearance of the gonopod, the most striking characteristic of the male, and its degree of development and differentiation in early stages were considered of the greatest interest and importance. A reliable criterion of sex in the early period of life of the fish was much desired.

The Gonopod and its Development.—The modified anal fin in the various groups of Pœciliids becomes a gonopod in accordance with a definite structural plan. The gonopod is formed by the modification of the third, fourth, and fifth anal-fin rays in the species whose anal fin possesses from six to ten rays. The general plan of the gonopod, with few exceptions, is as follows. The first and second ray are undivided and very short; the third ray is very much elongated; the fourth and fifth, also, are considerably elongated; while the sixth to the tenth rays are normal, bifurcate, and segmented. The third to fifth finrays, also, possess modified ossicles. (*See* Geiser, '23*b*.) Genera and species, while following this general foundation-plan, vary widely in the details of structure of the gonopod, as Langer ('13) has so beautifully shown. The primitive form of the gonopod occurs in *Petalosoma*. The apex of the gonopod may have a spoon-shaped process, the "prepuce" to aid in the transferrence of the sperm-balls to the genital papilla of the female during copulation. Such is the case in *Petalosoma, Pœcilia,* and *Molliensis*. Other modifications serving the same purpose are found in *Phalloceros,* in the form of hooks, and in *Phallotorynus* in the form of a curious structure resembling a garden trowel.

The writer's observations on the finer structure and progressive differentiation of the gonopod itself are reserved for future papers.

Hildebrand ('17) has called attention to the fact, also, that in young *Gambusia* the anal fins of the males and females are similar, and that the gonopod develops gradually, and at no definite age or length of the fish. When the young are only 13 mm. long and less than three months old the gonopod is sometimes developed: on the other hand, the fish may be 17 mm. long and five months or even a year old, and it is still not developed. A lot of 43 young of which he writes, born in May, 1914, were examined on October 15 of that year (when the smallest was 17 mm. long) and it was thought that they all were females, as no gonopod had developed. On June 3, 1915, however, six of the surviving fish were easily recognizable as males. The mortality was not stated, although he remarks elsewhere in his paper that the death-rate during early life in aquaria is inordinately high. From his results he concluded that the "modification of the anal fin into an intromittent organ may take place when the fish reaches a length of 13 mm., or at any later stage until it attains its maximum normal growth of about 25 mm."

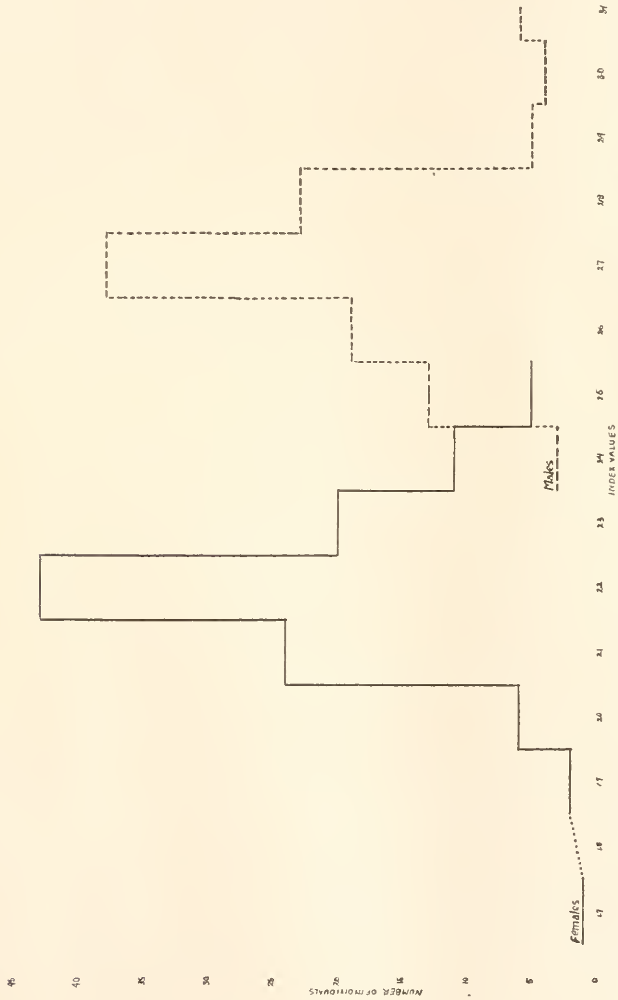
Mast ('21) as noted above, has given further evidence of the tardy development of the gonopod in the "sterile males" of *Gambusia holbrooki.* He found in these sterile males that the testes had already differentiated and were in a condition of advanced spermatogenesis. Even ripe spermatozoa were present in the form of sperm-balls. Yet with all this evidence of functional maturity, the anal fin gave no evidence of the sex of the individual.

The writer's studies show that the gonopod develops rapidly during the summer, so that by the time the season is over practically all the gonopods are developed and recognizable as such. The differentiation of the terminal portion of the gonopod into the characteristic hooks of, especially, the third, and the posterior branch of the fourth fin-rays does not, however, take place often until very late in the Fall or during the following Spring.

It is clear that this is hardly a criterion of sex that can be serviceable with younger fish of this species.

The Position of the Anal Fin as a Criterion of Sex.—In *Gambusia* the anal fin migrates forward in the male during the period of sex-differentiation with the result that when adulthood is reached the relative positions of the fin in the two sexes is quite dissimilar. The exact value of this "index," *i.e.,* the relation between the total length of the fish and the distance from the tip of the nose to the anterior border of the anal fin, is easily calculated. Measurements made of a large number of Pœciliid fish, embracing several genera, show that the adult index is always higher in the male, *i.e.,* the anal fin is placed relatively farther forward in the male. The mean index-value varies with the different species.

Graph I. shows the index values of two lots of *Gambusia* obtained from Beaufort, N. C., in 1920/21. While there is considerable variation, the indices for both sexes have very definite norms. As will be seen from the graph, in the males the class 2.7-2.799 is the mode; in the females, the class 2.1-2.199 is the mode. A comparison of the data shows that there is very little overlapping. Thus, in this population, only 3.6 per cent. of the males have an index-value of less than 2.500, while on the other hand, only 4.3 per cent. of the females show an index-value in excess of 2.500. The findings in the populations graphed are paralleled by the results of other series of measurements.



Graph I. Showing the mutual exclusiveness of the Index-values of an unselected population of adult male and female *Gambusia.*

From this we see that the indexes for adult male *Gambusia* are such that by means of them the male can easily be distinguished from the females, in the lack of better criteria, even with rather old "sterile males" in which the gonopod is not well developed. The indices have rather sharply-defined limits, and these are nearly mutually exclusive. Applying these facts to the examination of suspected male Gambusia, we find that the number of sterile males is inconsiderable and quite inadequate to explain the occurrence of unusual sex-ratios.

With young, immature *Gambusia,* however, the adequacy of this criterion of sex is extremely doubtful. In the case of young, if the gonopod has not commenced to differentiate, the only reliable method of ascertaining the sex of a given individual is by cytological examination.

After this digression on criteria of sex, we may resume discussion of methods.

Methods and Results.—During the spring of 1921 it was found possible in large indoor aquaria to raise young *Gambusia* and to keep them, with very little mortality until the males could be distinguished from the females. Four litters were raised as follows:

On May 15, 1921 four females, each approximately 45 mm. long, and well advanced in pregnancy were taken at random from a lot of gravid Gambusia which had been collected at Beaufort, N. C., March 22, and shipped to Baltimore. These were isolated in special breeding aquaria which consisted of ordinary 3.5-liter battery jars in which were hung cages made of wire netting of 3.5 mm. square mesh. The cages were coated with beeswax to prevent rusting. The gravid females were put into these wire baskets, one in each, and the aquaria stocked with plants as usual. When the young were born they darted out through the meshes of the mother's cage and were thus able to escape her cannibalism. Gambusia females very frequently eat their new-born young—a habit that is shared with some other viviparous cyprinodont fishes.

The females were fed a variety of foods: boiled white of egg, bread soaked in beaten egg and dried, and finely chopped snails. They devoured eagerly microcrustacea, enchytraeid worms, and mosquito larvea, but these food materials were not always available.

The four females designated *a, b, c,* and *d* had young on the following days: May 19, May 19, May 21, and May 21, respectively. Female *a* had a litter of 37 young; *b,* of 19; *c,* of 22; and *d,* of 24. The birth of all these litters except that of *a* was observed, and it is known with certainty that none of the young were eaten by the mothers. It is most probable in the case of Female *a* that none were eaten, for in the writer's stocks, 37 is a very good litter. As soon as parturition was completed the mothers were removed. The young were fed on Daphnia for a few days in the small aquaria where they were born; after which time they were removed and put into concrete aquaria 90×160×60 cm. These pools had been thoroughly cleaned out in the spring and stocked with *Elodea* and *Spirogyra.* During the summer the *Elodea* grew until it formed a thick forest of vegetation in the aquaria, offering excellent protection to the young Gambusia. Litter *a* was put into one of these concrete aquaria, while Litters *b, c,* and *d* were combined and put into another. They were left in these aquaria without food, except such as came to them in the form of insects, etc., until the first group was 26 days old, and the second group three and a half months old. They were then removed, killed, and fixed in modified Bouin's fluid. All of the 37 young in Litter *a,* and 58 of the 65 of the other litters, were recovered. In the aquarium containing the latter there were found in addition to the 58 individuals, 12 which were relatively very small. These twelve were evidently offspring of some of the 65 individuals which had been put into the aquarium in May.

The proportions of the sexes in Litter *a* were learned by a histological study of the gonads of all the individuals. The young were taken after fixing was completed, the viscera dissected out in a mass, and these sectioned and studied. The gonads in all of these were differentiated to such an extent that the sexes could readily be ascertained by histological examination.

The sex of the young in the litters which were three and a half months old when killed could be readily be learned with certainty by examining the gonads under a binocular microscope. This was done in all of the specimens except those which had well-developed gonopods and were consequently unquestionably males. It was found that all the males in this lot had developed a gonopod, so that the presence or absence of the gonopod would have been a satisfactory criterion of sex in the three and a half months old fish. However, to avoid any possible mistake, through the existence of sterile males, the gonads themselves were examined.

In Litter *a* there were 19 males, 17 females, and one whose sex could not be ascertained because a mistake in technique lost the gonads during imbedding. The individual whose sex is uncertain, however, was probably a female, as such a notation was made in the dissection notes. In Litters *b, c, d,* of the 58 surviving fish 27 were males and 31 were females. For the total group of 94 young *Gambusia* whose sex was learned with certainty, there were 46 males and 48 females, a very close approximation to a 1 : 1 ratio.

It will be recalled that in Litters *b, c,* and *d* seven fish were not recovered, and presumably died. If half of these dead were males and half were females, the proportions of the sexes would then show a still closer approximation to equality. Even if all the dead were females, still the sex-ratio would not even begin to approach the great disproportion found in adults.

In these populations, taken all together, the percentage of individuals whose sex was unaccounted for is very low—only 8.3 per cent.—so that the approximate equality of the sexes is not accountable for on the basis of a differential death-rate in favor of the males. On the other hand, there is evidence to show that the converse condition is responsible for the slight numerical inequality of the sexes in the older litters.

Corroboration of the conclusion reached that the numbers of males and females in *Gambusia* at birth are approximately equal is found in the results obtained by the examination of populations from two large pools kept under conditions as nearly ideal as possible. *I.* Into one of these pools there were put in May, 5 pregnant females. About the middle of October 45 young and three of the parents were recovered. Of these 45 young, 21 were males and 24 were females, again a very close approximation to equal numbers of the sexes. *II.* In a pond known locally as the "Euglena Pond" all of the *Gambusia* had died during the winter of 1920. This pond was stocked in May, 1921, with 48 gravid females. In the following October 284 individuals were taken with a dipnet at random from the pond. Of these 284 *Gambusia,* 94 were males, 60 were females, and 130 were so small that the sex could not be ascertained by external observation. Both of these collections were from the same parental stock as the females whose litters were studied. One is impelled to conclude as a result of these observations that the great excess of females in the parental stock (nearly 8 to 1) does not represent the proportions of the sexes at birth, but must have been due to a greater mortality of the males during either the juvenile period or later.

The only careful piece of work that has hitherto been done in the attempt to ascertain the sex-ratio at birth in *Gambusia* is that by Hildebrand, and his results are vitiated by a high death-rate. He found that five months after birth, of an original litter of 46 fish, none had developed a gonopod; and at the age of approximately 13 months, only six of the surviving fish possessed a gonopod, thus giving a very low ratio, even if a possible very high death-rate, which he mentions, is taken into consideration. But, as has already been seen, the factors of food and temperature are particularly potent in determining the rapidity of sexual development and the production of young in these viviparous fish. This was shown clearly in the work of Schmidt ('19, '19a),[8] as well as by the writer's studies on duration of pregnancy and gonopod-development.

In confirmation of the correctness of the conclusion that the normal secondary sex-ratio in at least some Pœciliids is approximately 1 : 1, Henn's work ('16) may be brought forward. A total of 2,070 individuals of *Lebistes reticulatus,* the millions fish, was obtained in a single collection with a very fine-meshed net in the Barbadoes under the direction of Professor C. H. Eigenmann. The collection gave an approximately 1 : 1 ratio. In the lot there were 520 males and 630 females, besides 920 fish less than 10 mm. long, and too small to permit ascertainment of the sex by external examination. Henn says on the point of the sex-ratio that "it is quite certain that this count of males includes only members of that sex while a few of the smaller specimens regarded as females may really have been immature males. It will thus be seen that the sex-ratio, when an adequate collection is at hand, does not materially differ from that found in other fishes." He applies this conclusion to the *Pœciliidæ* generally. Schmidt's ('20) experimental litters of *Lebistes* had a ratio of males to females of 100:116.6 (total of 78 young) and his other data also show an approximate equality of the sexes. *Lebistes* possesses very many physiological and cytological characteristics in common with its close relative, *Gambusia.* It would be a singular thing if certain closely-related species of a compact family such as the Pœciliidæ possessed an anomalous ratio of the sexes at birth, as has been assumed for *Gambusia.*

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[8] Duration of gravidity in *Lebistes* at 25° is about one month; at 18°, more than three months. (Schmidt, '19*b,* p. 4.)

In a very recent paper Aida has given data which demonstrate a practically even sex-ratio in a Japanese fresh-water pœciliid fish, *Aplocheilus latipes.* On assembling his data for His " Experiments 7-10, 10*A* and *B,* 11*A* and *B,* 12, 14, 17-21 " (all data given) we find that he had a total in his experimental litters of 2,438 females and 2,400 males, a sex-ratio of 100 males to 103.9 females. This is very close to unity.

The Chromosomal Constitution of the Pœciliidæ and its Bearing on the Sex-Ratio.—It is unfortunate that the chromosomes of teleosts are so unsuited to investigation because of their small size, lack of individuality, and tendency to clump on the equatorial plate. It may be proper however, to consider in detail in this connection some genetical investigations whose results give strong evidence of the type of genetical constitution possessed by Pœciliids. Schmidt ('20) in breeding-studies was able to isolate a color-marking in a race of *Lebistes* which was transmitted through the Y-chromosomes. He undertoook crossing experiments with two types of males on one type of females. The " new " male type, which we will call " *B,*" possessed a brilliant dorsal fin-spot which was entirely lacking in the " old " type, " *A.*" Other color characters made the two types instantly distinguishable. Crosses were made of Type *A*♂ Type *B*♀ and Type *B*♂ Type *A*♀, and back-crosses were made with F_1 populations. Breeding records were kept, with copious notes (and frequently water-color drawings) of a large number of individuals (*e.g.,* the registered males of F_2-F_5 total 998.) It was found as a result of all these crosses that the dorsal fin-spot of Type *B* was carried through the male parent only. This fact was demonstrated very beautifully by Schmidt's experiments, and caused him to conclude that the genetic constitution of this fish is of the XX, XY type, and that the Y-chromosome carries the factor for fin-spot. Since this paper was written, Winge has (*22a, 22b*) further demonstrated both cytologically and genetically the XX, XY constitution of the Pœciliids, and has greatly extended Schmidt's genetical studies. Aida ('21) has shown by his breeding experiments, also, that another Pœciliid, *Aplocheilus,* also has an XX, XY genetic constitution.

If, indeed, the genetic constitution of the Pœciliidæ is of this XX, XY type, then it follows as a corollary that in the male, which is the heterozygous sex, approximately equal numbers of male- and of female-determining gametes are produced. Assuming that no differential chance-of-fertilization exists we would infer that in the young there would be nearly equal numbers of males and females. This is exactly what we do find under controlled conditions. If then in the adult there is a pronounced excess of females it would appear that this excess must be the end-result of a differential death-rate of the sexes.

A Differential Death-Rate in Gambusia.—The males are much less resistant to harmful environmental factors than the females, and hence have a lower survival value. This is shown by the results of several lines of experiment and observation.

In those cases where quantitative studies have been made with analyzed factors, the males do not survive as well as the females. Thus in the writer's experiments, it was found that high temperatures, high H-ion concentration, oxygen-deficiency, and concentrations of KCN kill the males much more readily than the females. For example, in a collection of 283 young *Gambusia* which was killed in hot water, practically all the fish that died first were males. Bellamy (*in lit.*) states that in his experiments with other Pœciliids with high temperatures, oxygen deficiency KCN, etc., he obtained results that are "in complete agreement" with the writer's contention that "males are more susceptible to 'difficult' conditions than the females."

Unanalyzed deleterious influences in the environment also bring about a lethal selection to which the males succumb more readily than the females. The writer showed (Geiser, '21*a*), for example, that male *Gambusia* were less resistant than females to disturbances incidental to shipment, during both cold and warm weather. Thus, in cold-weather shipments the male death-rate was one and one half times the female death-rate, and in warm weather shipments, two and one half times the female death-rate. This latter result, also, was obtained when the females were heavily gravid. In aquarium catastrophes, such as epidemics of *Icthyriopthirius*- and *Saprolegnia*-infestations, the males suffer much more severely than the females. This experience of the writer is confirmed by that of European aquarists generally. In catastrophes of unknown cause, the same holds. Thus, to record one example out of many recorded in my note book: "On 16 November, 1921, in aquarium containing 94♀, 20♂, 16 dead♀ and 14 dead ♂ were found. No cause was ascertained. No fungi. Life-conditions apparently excellent. Death-rate for males 800–, and for females 148.8– per thousand, *i.e.*, 5 3/8 to 1."

The greater ability of the females to survive is moreover evidenced in the proportions of males and females which in the writer's aquaria survive the winter. Thus, *e.g.*, in October, 1921, approximately 100 Gambusia, fairly equally divided as to sex, were left outdoors in a somewhat protected concrete aquarium to pass the winter: on April 10, 1922 all the survivors, 32 in number, were recovered. Of these, only one was a male. It is consequently evident that the females were more resistant to the weeding-out process than the males, for only 40 per cent. of the females, as compared with nearly 100 per cent. of the males, had died.

There is still another way in which the numbers of males in a given lot of *Gambusia* are reduced. The males are smaller and hence are more liable to be devoured by small predaceous fish than the much larger females. Gravid female *Gambusia* in aquaria, also, attack and frequently kill the males. Records kept of the sex of dead fish taken from the aquaria show over ten times as many males as females. This fact lends support to Carbonnier's ('66) contention (Darwin, '75, p. 335) that the males suffer from their small size since they are liable to be devoured by females of their own species.

It is thus apparent that in *Gambusia* there is a differential death-rate, and that its operation explains the excess of females found in adult populations.

Elsewhere the writer (Geiser, '23*a*, '24) has shown that such a differential death-rate obtains in various fishes, crustacea, insects, for man, and for mammals other than man. Among the fishes besides those already mentioned it has been quite clearly demonstrated in the European Plaice, the Canadian Plaice, Witch, British Salmon, Smelt, and Dogfish, and the Japanese Sweetfish or Ayu. The reader is referred to these papers for the evidence, which conclusively demonstrates that in many diverse groups of animals the male is less viable than the female.

IV. Conclusions.

1. Field collections of *Gambusia* almost invariably possess a great preponderance of females.
2. These sex-ratios vary with the different seasons of the year.
3. Studies on the spermatogenesis of *Gambusia* fail to reveal any unusual distribution of the chromosomes which would explain the atypical sex-ratios found.
4. Experiments with *Gambusia* raised in aquaria show the proportions of sexes at birth to be approximately equal.
5. The males have a higher death-rate than the females, thus causing the atypical sex-ratios found in the adult populations.

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EXPLANATION OF THE FIGURES.

(All figures were drawn at table level with the aid of a camera-lucida, using a 1.8 Bausch & Lomb Fluorite oil immersion with a Zeiss No. 12 compensating ocular at a tube-length of 160 mm. The drawings thus obtained were enlarged 1.75 diameters with a pantagraph. These final drawings were reproduced without reduction.

PLATE I.

FIGS. 1–2. Dividing spermatogonia from the peripheral portion of the testis.

FIGS. 3–5. Spermatogonia in multiplication-divisions in the cyst.

FIGS. 6–7. Stages later than the preceding, showing reconstitution of the nucleus after spermatogonial divisions.

FIGS. 8–10. Final spermatogonia.

FIGS. 11–16. Leptotene stages.

FIG. 18. Pachytene.

FIG. 19. Cell in the pachytene stage, cut transversely to the chromatin threads.

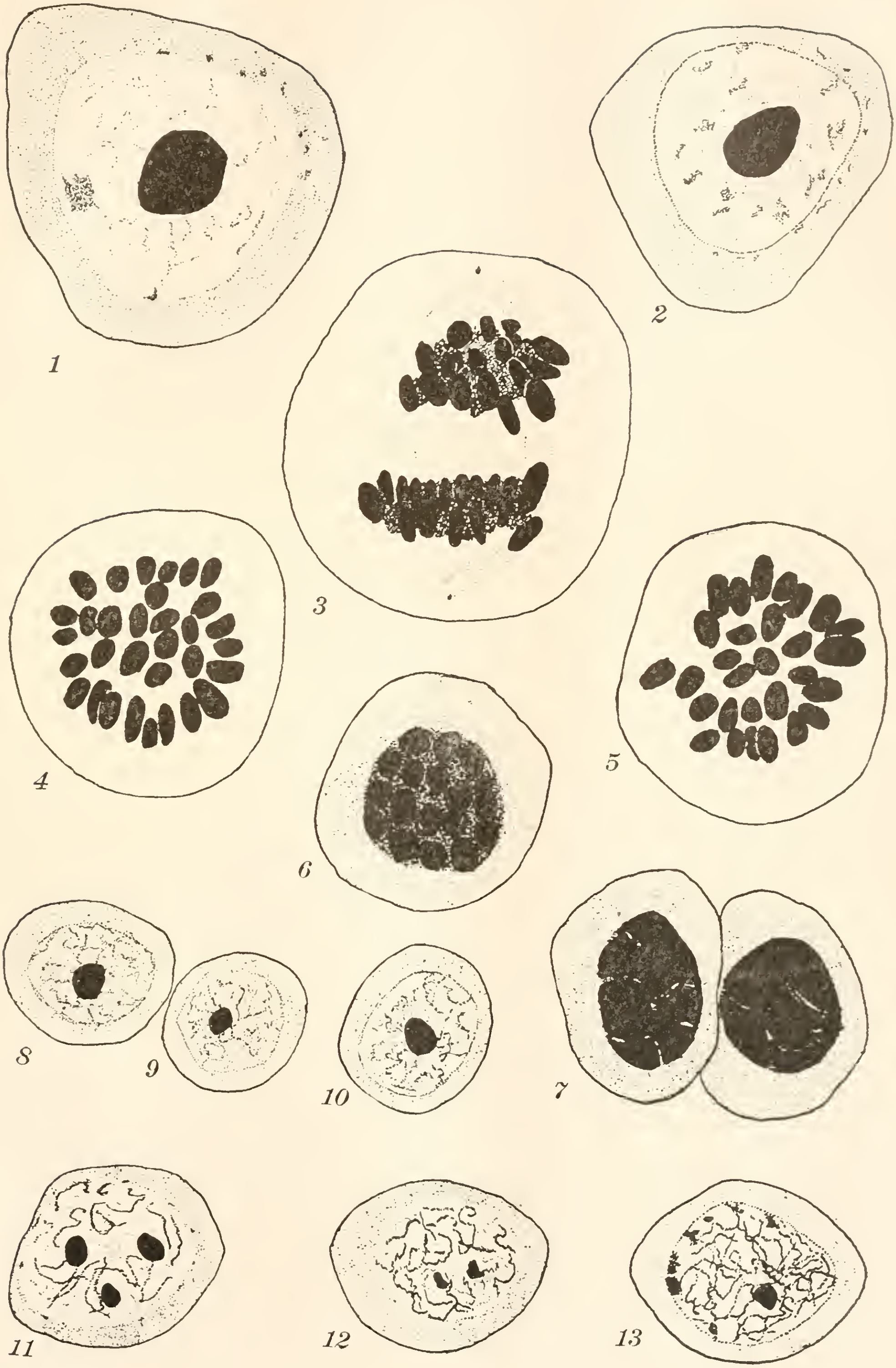
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Plate II.

Figs. 20–21. Tetrads.

Fig. 22. Diakinesis.

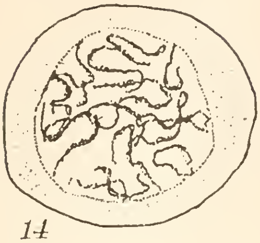
Fig. 23. Tetrads grouping to form an equatorial plate.

Figs. 24–25. First-spermatocyte divisions, viewed from the side.

Fig. 26. Metaphase plate of second-speratocyte division.

Fig. 27. Fusion of chromosomes after completion of the second-spermatocyte division.

Figs. 28–30. Stages in the reconstitution of the spermatid nucleus.



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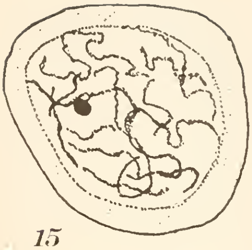

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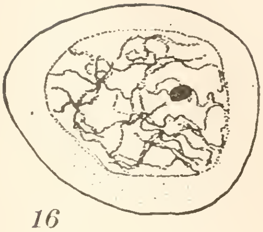

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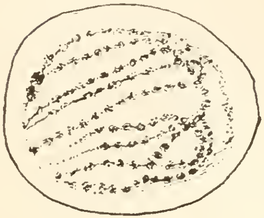

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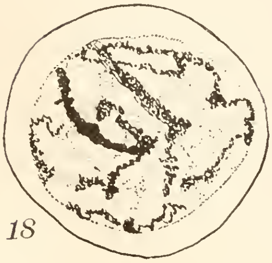

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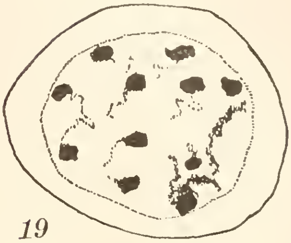

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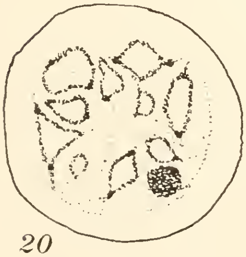

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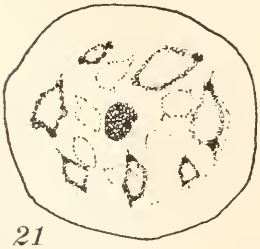

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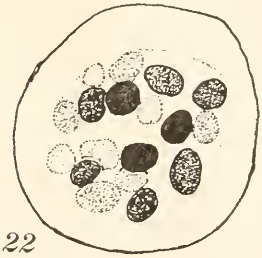

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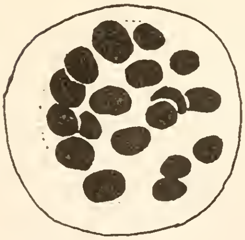

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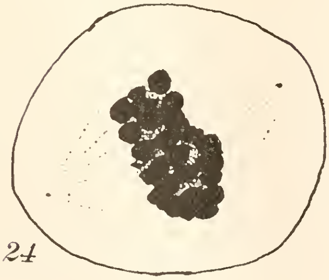

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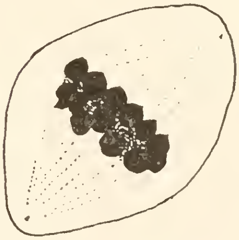

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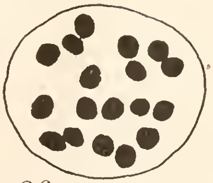

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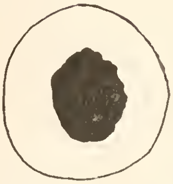

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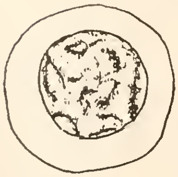

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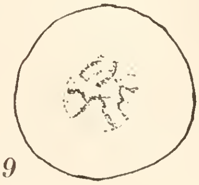

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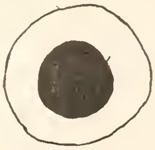

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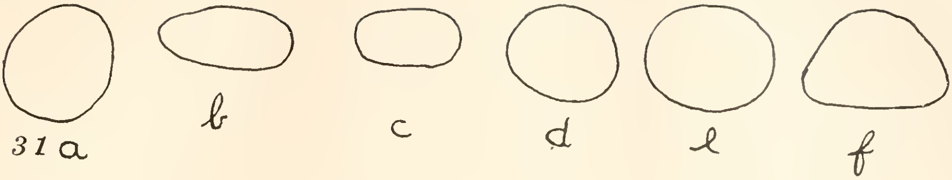
PLATE III.

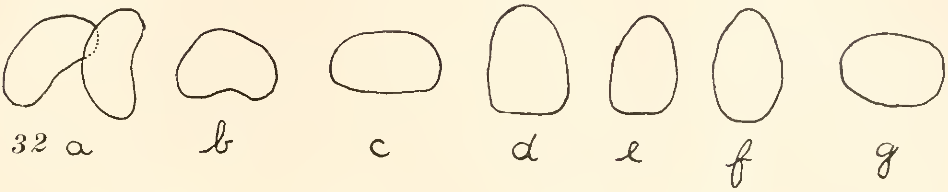
FIGS. 31–33. Outline drawings showing the forms assumed by the chromatin in the development of the head of the spermatozoon.

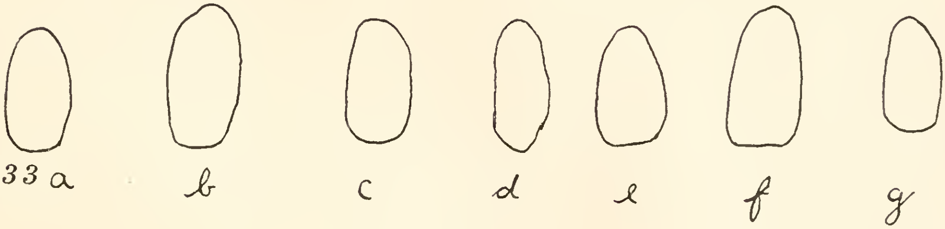
FIGS. 34–35. Outline drawings showing the developing spermatozoa arranging themselves at the periphery of the spermatozeugma that is forming.

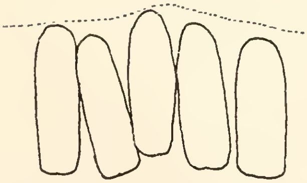
FIG. 36. Tangential section of the wall of a spermatozeugma, showing the closely-packed arrangement of the spermatozoa in the spermatozeugma.

FIG. 37. Spermatozoa from a nearly-ripe spermatozeugma.









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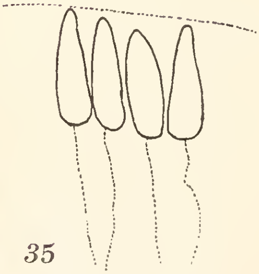

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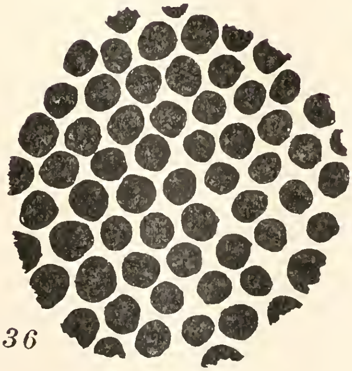

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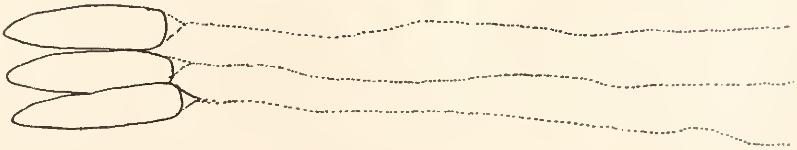

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Vol. XLVII October, 1924 No. 4

BIOLOGICAL BULLETIN

CAN THE EARTHWORM PHARYNX[1] EPITHELIUM PRODUCE CENTRAL NERVOUS TISSUE?

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It has long been known that, when several segments of the head end of an earthworm are removed, within a comparatively short time a new head will be regenerated. The first of the new tissues to be differentiated is the nervous tissue. Hescheler in 1898 described in detail the regeneration of the nervous and other organs in several species of earthworm. He believed the mass of " Regenerationsgewebe " from which the nerve cells differentiated to be largely of epidermal origin. This would seem to be the natural source of nerve material, since in the embryo the nervous system is formed by a thickening and infolding of the ectoderm.

The operation in which several head segments are removed involves loss to all of the tissues of that region. Consequently regeneration is an extensive process in which all of the injured tissues must be more or less concerned. But if the cephalic ganglia, or "brain," be removed through a small incision in the body wall, the operation being carried out so that no tissue other than nervous is removed and with a minimum of damage to non-nervous tissues, the resulting regenerative situation is much simpler. The non-nervous tissues will require only a direct healing of the wounds caused by the incision. If, under these circumstances, the brain is regenerated, what will be the source of its material?

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[1] The region of the alimentary canal referred to here and throughout the paper is so precisely at the junction of the buccal cavity and the muscular pharyngeal region as to occasion doubt whether its epithelium should be designated as buccal or pharyngeal. It is very slightly anterior to the heavy dorsal muscle of the pharynx. Transverse sections through the brain fundament of worms regenerating after an operation of the kind described in this paper invariably include the " Schlundcommissuren," and the " Schlundganglien " or " Schlundgeflecht " (Vejdovsky, 1884; and other authors) consisting of heavy ganglionated nerves passing from the commissures to the wall of the alimentary tube. " Schlund," at least for the Lumbricidæ, must have the sense of pharyngeal. The term "circum-esophageal commissures," commonly used in English texts, is inappropriate for Lumbricidæ because the commissures are remote from the esophagus. Vejdovsky (1884, p. 81) says: " Die Schlundcommissuren umgreifen den Pharynx. . . ." In view of these facts we designate the region in question as pharyngeal rather than buccal.

Friedländer (1895) carried out operations of the latter sort. He regarded the regenerated supra-esophageal ganglia as derived mainly from the old nerve tissue, but perhaps partly from cells of the wound tissue or "Regenerationsgewebe." He suggested that these latter cells might be of leucocytic origin. Hübner (1902) repeated the experiment and concluded that leucocytes played little or no part in the regeneration and that the epidermis was mainly responsible for it. His figures show a mass of cells, with small deeply stained nuclei, lying below the epidermis in the region of the wound and extending down to the region of the developing brain. But his figures and his scanty description do not afford convincing evidence that these cells are epidermal in origin and are actually contributing to the new brain fundament. Some time ago the senior author found, in sections of earthworms regenerating after an operation of this kind, indications that the pharynx[1] epithelium might contribute cells to the regeneration of the nervous organs of the head. Accordingly the study described below was undertaken, partly for the purpose of obtaining more complete data concerning the histogenesis of the brain following an operation in which no head segments are removed, and especially with a view to ascertaining whether pharynx epithelium can produce nerve cells. Involved in the latter question is another—will an epithelium which is not itself injured become active and participate in regeneration?

A wound is supposed to supply the stimulus for regeneration. Therefore, with reference to the question as to whether an uninjured epithelium will engage in regeneration, the ideal operation would have been to remove the brain without causing any other injury to the worm. This being obviously impossible, the following plan was devised. After narcotizing the worm in 0.2 per cent. chloretone for ten minutes, an incision was made in the dorsal body wall, in or near the median plane, and extending from the seventh segment forward to the third. The remainder of the operation was performed under a compound dissecting microscope. Very fine oculists' scissors were used. The body wall was pinned back, exposing the cephalic ganglia lying just above the pharynx in the third segment. With dissecting needles the ganglia could be raised from the tissue below and then snipped off at either side. The operation offered considerable difficulty, so that sometimes a part of either connective with the heavy nerve which runs to the pharynx wall was removed. When this happened it was impossible to tell whether or not the pharynx was injured, but it is very likely that it was. Therefore, to obviate possible injury to the dorsal region of the pharynx wall, and in order that the epidermal wound be as remote as possible from the expected (dorsal) site of the brain anlage, the operation was in some cases modified by making the incision dorso-laterally or even sometimes ventro-laterally.

Operations of the kind described above were performed on about eighty worms. The species *Allolobophora fœtida* was used because of its capacity for quick regeneration. At various stages the operated worms were fixed in Bouin's picro-formalin, the anterior ends cut transversely, and the sections stained with hæmatein-eosin. The following description is based on a careful study of twenty-five cases.

To insure appreciation of conditions in the operated animals, reference must be made to certain features of the brain region of the normal earthworm. Fig. 1 represents diagrammatically a typical cross-section through the brain region of the normal worm. The epidermis (*e*) consists of fairly large columnar cells having oval nuclei each containing a prominent nucleolus, and among these cells are numerous large gland cells. Beneath the epidermis is the body-wall muscle (outer circular and inner longitudinal fibers) and between the inner muscle layer and cephalic ganglia (*br.*) is a space containing some connective tissue and blood vessels (*bl.v.*). Around the brain is a distinct connective tissue sheath (*c.t.s.*). The periphery of the brain tissue is largely made up of typical pear-shaped nerve cells, while the central mass is mainly fibrillar. Continuing from the brain downward around the pharynx, to which they send off heavy nerves (*p.n.*) on either side, the circum-esophageal commissures (*c.c.*) merge into the sub-esophageal ganglion (*s.g.*). (The section drawn is slightly anterior to the point of union of the commissures.) The pharynx wall consists of an epithelium (*p.e.*) surrounded by a thin layer of muscle and connective tissue (*n.p.*). The epithelium itself (Fig. 2) is composed of columnar cells with oval nuclei, most of which contain a prominent nucleolus. They are, indeed, very similar to the cells of the epidermis, perhaps being a trifle smaller. A point to be particularly emphasized is that *the nervous tissue of the brain is bounded on the side toward the pharynx by enveloping layers of non-nervous tissue, that the pharynx epithelium is bounded on the side toward the brain by layers of non-nervous tissue, and between the two organs is some free space.* Nowhere is nerve tissue in intimate association with the pharynx except where the lateral nerves join it.

Within a few hours after the operation the wound closes over, and only a scar marks the region in which it was made. Cross-sections of an early stage of regeneration, one week after the operation (Fig. 3), show the epidermis entirely healed, but the region of the wound (*w.*) is readily distinguishable through the absence of the characteristic gland cells. Below the epidermis, extending down through the cut muscle layers, across the cœlomic space and joining the pharynx wall, is a dense mass of deeply stained cells (*r.t.*). Fibers extending from the cut end of one commissure pass through this cell mass just above the pharynx to the cut end of the other commissure. There is little doubt that these are nerve fibers and that they are outgrowths of cells of the old nerve tissue. Lying approximately mid-dorsally and exactly in the pathway of these fibers is a small and not definitely limited group of cells (*br.f.*) made conspicuous by their especially intensive staining. These cells, containing large nuclei with prominent nucleoli, are similar to the cells of both pharyngeal and epidermal epithelia. Their position, corresponding precisely to that occupied by the obvious brain fundament of later stages, as well as their character, marks them as the early brain fundament. (The sections in this series were not exactly transverse. The section represented in Fig. 3 contains a slightly lateral portion of the brain fundament.) There is no line of demarcation between the brain fundament and the pharyngeal epithelium. One merges uninterruptedly into the other.

Fig. 4 is a high-power drawing of that region of the pharyngeal epithelium marked *X* in Fig. 3, together with the neighboring portion of the brain fundament. The connective tissue and muscle which in the normal pharynx lie external to the epithelium (Figs. 1 and 2, *n.p.*) have entirely disappeared from this region of the pharynx wall. Not only has the dorsal pharynx wall lost its external layer, but its epithelium shows signs of considerable activity. Instead of being composed of tall columnar cells with fairly well-defined walls, the epithelium has become syncytial. Its nuclei are larger than the normal epithelial nuclei and more nearly spherical, but, like the normal, show the usual prominent nucleolus. Two mitoses appear in the part of the section drawn (Fig. 4, *m.*) and numerous nuclei of the epithelial type lie in more or less deep positions. The whole appearance of this syncytial cell mass is indicative of rapid cell proliferation.

In sharp contrast with these conditions are those found between the brain fundament and epidermis (see Fig. 3). The distance between the brain fundament and the epidermis is very much greater than that between the brain fundament and pharynx epithelium. The mass of cells between brain fundament and epidermis consists mainly of cells with small deeply stained nuclei without prominent nucleoli, resembling therefore neither the epidermal nuclei nor those of the brain fundament. They remind one rather of leucocytes. Here and there within this mass is found an occasional nucleus which is similar to those of the epidermis. No mitosis could be observed in the epidermis. The presence of mitosis at this stage in the pharynx epithelium and its absence in the epidermis is significant.

At a twelve-day stage of a case in which the wound was made dorso-laterally, the brain fundament approaches normal form, consisting of two enlargements connected mid-dorsally. The position of the wound is readily distinguishable by the absence of differentiated epidermal gland cells. The cut through the muscle layers is now completely healed, but its location is marked by the presence of more than the usual number of nuclei. There is no mass of wound tissue extending from the healed epidermis down to the pharynx as there was in the seven-day stage. Some of the nuclei in the region where the muscle layers have been repaired resemble epidermal nuclei, but no mitosis can be observed in the epidermis. Lying about midway between the epidermis and pharynx epithelium is the brain fundament. Fig. 5 represents the median region of the brain fundament and the neighboring pharynx epithelium. Between the brain fundament (*br.f.*) and the pharynx epithelium (*p.e.*) occurs a compact cell mass similar to that found in a corresponding place in the seven-day stage. The dorsal pharynx epithelium in this worm is still active. The non-epithelial tissue of the pharynx wall terminates on either side, leaving the mid-dorsal region of the epithelium in close relation to the new brain. Throughout this series of sections mitoses (*m.*) are fairly abundant in that part of the dorsal epithelium lying beneath the brain fundament, but not elsewhere. The occurrence of these mitoses, together with the presence of nuclei precisely like those of the epithelial layer, but sub-epithelial in position (Fig. 5, *s.n.*), affords the best possible evidence that the pharynx epithelium is proliferating cells which are passing into the region of the regenerating brain. Smaller nuclei, probably leucocytic in nature, are found interspersed with these other nuclei. Some fibrous material (*f.*), probably indicating the reëstablishment of the muscle and connective tissue layer of the pharynx wall, and also blood vessels (*bl.v.*), are observed between the pharynx epithelium and the brain.

In a nineteen-day stage the new brain has a normal shape, but is not yet of normal size. Very little evidence of the wound remains aside from the absence of differentiated gland cells in the mid-dorsal epidermis. The wound in the muscle layer is barely recognizable. Between the brain fundament and the pharynx epithelium lies a compact cell mass as in earlier stages, but with some indications of the establishment of the non-epithelial tissue of the pharynx wall. Numerous cases of mitosis occur in the dorsal pharyngeal epithelium. Several cases of mitosis within the brain fundament demonstrate that the cells already there are increasing in number.

In order to put the epidermis at the greatest possible disadvantage as a source of material for the dorsal brain anlage, a number of operations were performed in which the incisions were made either laterally or ventro-laterally. If, now, the epidermis is to provide cells for the regenerating brain, such cells must arise from the uninjured mid-dorsal epidermis and pass through the muscle layers to the region where the brain should develop, or else they must migrate there from the lateral epidermal wound. Either because of the severity of this operation or because of the weakened condition of worms kept through the winter, very few of these worms lived. One of the survivors was fixed after ten days' regeneration. In sections (Fig. 6) through the brain region of this worm the lateral location of the wound (*w.*) is recognizable by a break in the muscle wall and the lack of differentiated gland cells in the overlying epidermis. The wound is remote from the brain fundament (*br.f.*) which is already clearly established in its normal mid-dorsal position. While the break in the body wall is filled with regeneration tissue (*r.t.*), this tissue does not extend across the cephalic space to the brain fundament. In this operation the lateral nerves were left intact and there is no evidence that the pharynx epithelium was injured at any place. Nevertheless, as regards relation of brain fundament to the pharynx wall, we find conditions here the same as in cases described above—an undoubted activity on the part of the dorsal pharynx wall, as evidenced by its lack of the external connective tissue and muscular layer and by continuity of the proliferating epithelium (*p.e.*) with the regenerating brain (*br.f.*). The brain anlage appears as a mass of deeply stained cells lying mid-dorsally in the course of a tract of fibers which have grown across between the two commissures. Most of these cells are of the characteristic nervous and epithelial type. Interspersed with these are smaller nuclei of doubtful significance, probably leucocytes and connective tissue elements. Since in this case the epidermis as a source of new brain material is eliminated, there remain two possible sources—the pharynx epithelium and the old nervous tissue. However much the latter may supply to the new brain fundament, it is certain that the pharynx epithelium plays an important part.

While the evidence in a problem of this sort is necessarily indirect, since one can not watch the process of regeneration within the living worm, but is compelled to rely on the study of fixed and sectioned material, nevertheless, in the twenty-five worms which were carefully studied, the following facts stand out significantly:

1. While there is evidence in some cases that the epidermis is contributing cells to the regenerating brain, there is much more evidence of the same kind that the pharynx epithelium is contributing.

2. In nearly all cases the brain fundament is in closer relation to the pharynx epithelium than to the body wall.

3. An uninjured dorsal epidermis does not become active and contribute to the new brain, while an uninjured dorsal pharynx epithelium does.

4. When the incision is made decidedly laterally, there is no evidence of migration of cells from the wounded epidermis to the new brain.

5. It is certain that the old nerve cords contribute some material to the regenerated brain. Just how much it is impossible to say.

6. The cells which seem to be passing from the pharynx epithelium into the brain fundament have nuclei precisely like those of cells which, in the later stages of regeneration, are certainly to be identified as neuroblasts.

If, as all the evidence described above clearly indicates, the pharynx epithelium of the earthworm plays a large part in the regeneration of a central nervous organ, we have here the phenomenon of a tissue specialized for one purpose giving rise to one highly specialized for another and very different purpose. Still more remarkable is the fact that the pharynx, which in ontogeny has nothing to do with the origin of the nervous organs, should, upon removal of the brain, become an important source of material for its regeneration. The only morphogenetic relation between the old nerve tissue and the pharynx epithelium is that both are ectodermal in origin, since the latter develops by invagination of ectoderm.

This case resembles very closely the regeneration of the salamander's lens as demonstrated by Wolff's (1894, 1895) experiments on Triton. The lens, which in ontogeny is formed by a thickening and invagination of ectoderm, is regenerated from the upper edge of the iris. It can hardly be imagined that, in nature, an earthworm accidentally loses a brain or the salamander a lens.

Therefore the tissues concerned in regenerating these structures could hardly have acquired that power through natural selection. In both cases the source of the regenerating material is an epithelium which, originally ectodermal, has come to constitute a specialized part of an internal organ and yet has not lost its primitive ectodermal potentialities, or "totipotence."

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ABBREVIATIONS.

bl.v.	Blood vessel.
br.	Cephalic ganglia ("brain").
br.f.	Brain fundament.
c.c.	Circum-esophageal commissure.
c.m.	Circular muscle layer.
c.t.	Connective tissue.
c.t.s.	Connective tissue sheath.
e	Epidermis.
f	Fibers.
l.m.	Longitudinal muscle layer.
m.	Mitosis.
n.p.	Non-epithelial (muscular and connective tissue) layer of pharynx wall.
p.e.	Pharynx epithelium.
p.n.	Pharyngeal nerve.
r.t.	Regeneration tissue.
s.g.	Sub-esophageal ganglion.
s.n.	Sub-epithelial nuclei.
w.	Region of wound in epidermis.

EXPLANATION OF FIGURES.

1. Diagrammatic cross-section through the brain region of a normal earthworm. × 50.

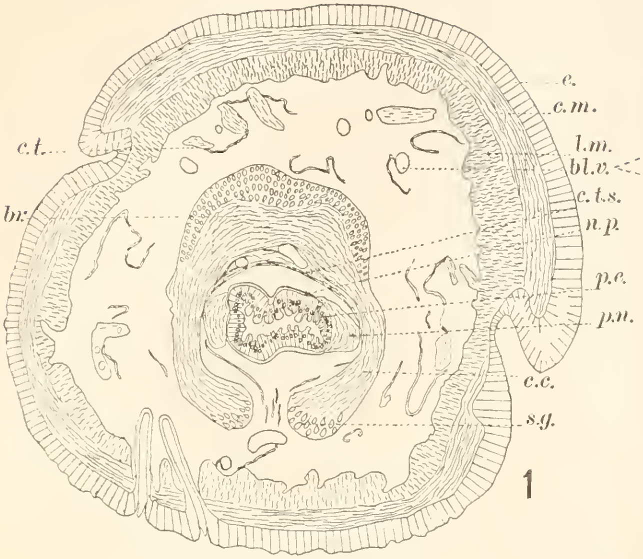
2. Section of mid-dorsal pharynx wall in the brain region of a normal worm. × 250.

3. Diagrammatic cross-section through the region of the regenerating brain 7 days after the operation. The section drawn was not precisely transverse and shows a slightly lateral portion of the brain fundament. × 50.

4. High-power drawing of that portion of Fig. 3 indicated by *X*. × 300.

5. Section through the mid-brain region of a worm at the 12-day stage of regeneration, together with the pharynx epithelium lying immediately below that region. × 300.

6. Diagrammatic cross-section through a worm at the 10-day stage of regeneration. The incision was made in a lateral position (*w.*). × 40.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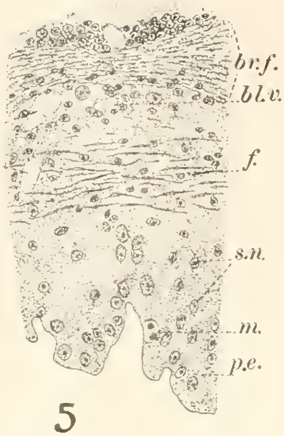
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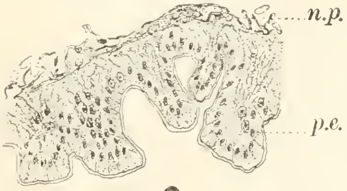

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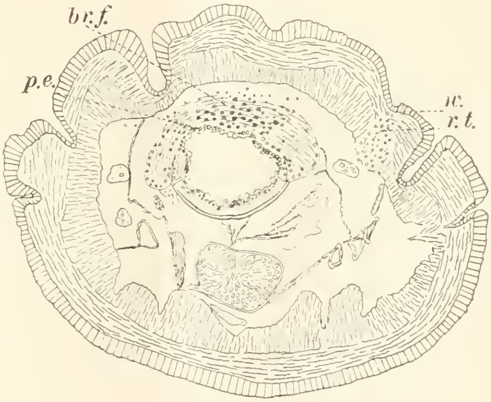

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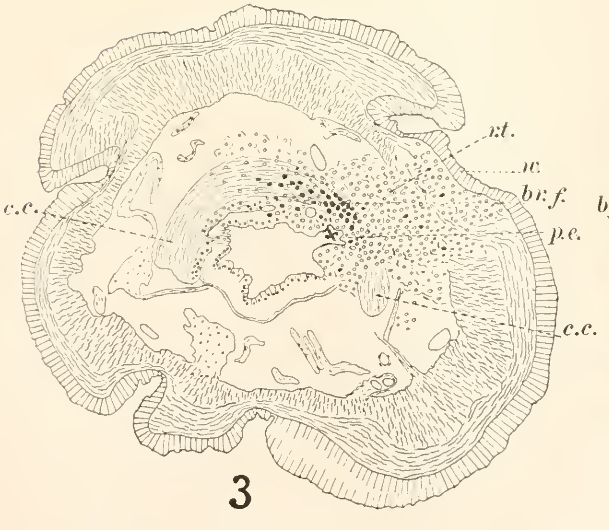
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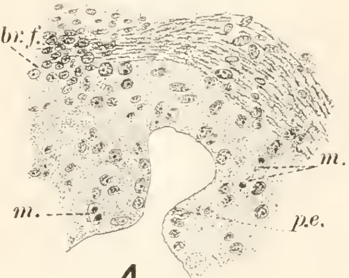

4

MIRIAM F. NUZUM AND HERBERT W. RAND.

BOTRYLLUS SCHLOSSERI (PALLAS): THE BEHAVIOR OF THE LARVA WITH SPECIAL REFERENCE TO THE HABITAT.[1]

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UNIVERSITY OF MAINE.

The purpose of the work reported in this paper was to determine more accurately than had been possible previously, the nature and effect of certain of the responses of the larva of *Botryllus,* and to investigate more fully the relation they bear to the apparent " selection " on the part of the larva of the place for attachment preparatory to metamorphosis. The work was done during the summer of 1923, at the Marine Biological Laboratory, Woods Hole, Massachusetts.

Thanks are due to the university of Maine for making the work possible, to the Marine Biological Laboratory for the facilities for the work, and in large measure to Dr. Caswell Grave for his helpful suggestions and generous assistance.

During the course of previous experiments, some evidence appeared indicating that the *Botryllus* larva tends to " select " definite places of attachment for metamorphosis. Larvæ were allowed to undergo metamorphosis in crystallization dishes containing blades of eel grass stretched obliquely from the bottom of one side of the dish to the top of the other, and held in place by glass slides. When sufficient time had elapsed for metamorphosis to take place, the location of the zooids was noted. Measurements of the eel grass, the diameter of the dishes, and the depth of water were made in each case, and calculations were made to determine the total area available to the larvæ for metamorphosis, and the percent of that area which was offered by the eel grass. Similarly, the percentage of larvæ metamorphosing on the eel grass was determined. The results of six such experiments—including the four previously reported—are indicated in the following table.

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[1] This paper is supplementary to one previously written by Dr. Grave and myself on the same species of *Botryllus.* It may be found in the *Journal of Morphology,* Volume 39, Number 1.

TABLE SHOWING THE RESULTS OF SIX EXPERIMENTS DESIGNED TO DETERMINE WHETHER OF NOT THE LARVÆ "SELECT" EEL GRASS AS A PLACE FOR METAMORPHOSIS.

Experiment.	Per Cent. of Metamorphosing Zooids Attached to Eel Grass.	Per Cent. of Total Area Offered by the Eel Grass.
1	55.8	11.1
2	48.1	18.3
3	55.2	20.0
4	43.7	19.4
5	12.0	14.4
6	32.8	17.6
Average	41.23	16.8

These results indicate that the larvæ under these laboratory conditions, tend to "select" eel grass as a place for permanent attachment and metamorphosis. It seems safe to assume that the responses of the larva which tend to bring it into contact with eel grass under the conditions in the experiment, would function in a similar way in the natural habitat of the larva. Granted, then, that a certain amount of "selection" does take place in determining the place of metamorphosis, what are the specific responses of the larvæ—and what the stimuli bringing about these responses—whereby the larva is led to a place suitable for metamorphosis? Does the eel grass offer any chemical attraction for the larvæ? Does the larva show a positive response to gravity during the latter part of its free-swimming period, thus in its natural habitat coming in contact with the lower parts of the eel grass blades? Is the negative response to light responsible for this "selection"? Does the larva tend to follow surfaces, thus keeping it in a favorable locality once it has reached it? Does the response of the larva in swimming upward when renewing activity play any part in determining its place of attachment and metamorphosis? These are some of the questions which suggested themselves. Enough of them have been answered to suggest at least which factors function in determining the place of metamorphosis of the larvæ.

Chemical Attraction.—In considering the question of the "selection" of the eel grass by the larvæ, it seemed at first quite possible that the eel grass might give off some substance to which the *Botryllus* larva orients. The result of one experiment, however, indicated plainly that this was not the case. Clean blades of eel grass were stretched between the ends of a piece of coarse wire netting, bent into the shape of a flat U. The blades were so arranged that their flat surfaces were parallel to the central portion of the wire netting. This apparatus was suspended near the bottom of a large aquarium at the least illuminated side. The only source of light was an opening in the covering of a north window of approximately the same size as the aquarium. Since the aquarium stood immediately in front of this opening, the intensity of the light on the two sides of the eel grass blades was practically the same, and the possibility of light intensity playing any part in determining the location of the larvæ on the eel grass was thus largely eliminated. Larvæ were introduced into the aquarium immediately after liberation from the parent colony, and were allowed to metamorphose there. When sufficient time had elapsed for attachment to have taken place, records were made of the location of the zooids. It was expected that if the eel grass gives off a chemical substance to which the larva orients, at the time when metamorphic changes are about to begin, the metamorphosing zooids would be found on the eel grass. Although larvæ had been observed to swim about the grass blades, not one metamorphosing zooid of the several hundred in the aquarium, was found attached to them. This result indicates plainly that chemical attraction is not a factor in determining the place of attachment of the larvæ for metamorphosis.

Response to Gravity and Light at the End of the Free-swimming Period.—In the experiments first described in which larvæ were allowed to metamorphose in crystallization dishes containing blades of eel grass stretched from the bottom of one side to the top of the other the factor of gravity would have but a small part in determining the place of metamorphosis of the zooid because of the shallowness of the water. It seemed as if in its natural habitat, a positive response of the larva to gravity at the time of metamorphosis would be advantageous, since it would tend to bring the larva into contact with a place suitable for metamorphosis, such as the lower portion of eel grass blades. Previous work, however, had shown that although on casual observation larvæ appeared positive to gravity at the end of the free-swimming period, the response of the larva was not as simple as at first considered. After experiments of many types had been performed, the conclusion was reached that at the end of the free-swimming period, larvæ are indifferent in their response to the stimulus of gravity. One experiment is sufficient to illustrate. Larvæ just liberated from the parent colony were allowed to metamorphose in a cylinder of 500 c. c. capacity held in an oblique position at an angle of about 45 degrees. The effect of light was eliminated in one case by jacketing the cylinder completely with black paper; in the other three, by performing the experiments in a dark-room. After the time necessary for metamorphosis had elapsed the cylinders were examined. The attached zooids were found evenly distributed from the top to the bottom on the lower side of the cylinder in three of the four cases; in the fourth, zooids were much more frequent near the top of the cylinder than the bottom, but as in the other three cases, they were much more frequent on the lower than the upper side. If the larvæ are positive in their response to gravity at the end of the free-swimming period, it was to be expected that the metamorphosing zooids would be found attached to the lower end of the cylinder. The fact that larvæ were not found in large proportion at the bottom of the cylinders, shows clearly that they are not positive in their response to gravity at the end of the free-swimming period. Their even distribution would indicate that the larvæ pass thru a long period of indifference to gravity during which, in the absence of light, random movements send them to all parts of the container, and that when metamorphic change sets in, the larvæ become inactive, sink, and attach themselves to the first surface with which they come in contact.

The question which naturally follows, is why, in the absence of a positive response to gravity, the larvæ metamorphose at the bottom of a cylinder held in a vertical position, as has been shown to be the case in a former paper. There are two possible explanations. If the larvæ attach and begin metamorphosis on the surface with which they first come in contact when swimming movements cease, as appears to be the case when confined in a glass cylinder, the bottom of the container must of necessity become the place of attachment of a large proportion of the zooids present in it. The remainder which metamorphose elsewhere get caught at the top of the water or near small bubbles of air on the side of the cylinder by surface tension, and still others, in sinking strike the sides of the container, and attach before reaching the bottom. A second explanation for the occurrence of metamorphosis at the bottom of a container standing in a vertical position, may be found in the fact that the larvæ are negative in their response to light at the close of the free-swimming period. Two experiments will serve to illustrate. Larvæ just liberated from the parent colony were allowed to metamorphose in a cylindrical graduate placed vertically, the sides of which had been partially covered with black paper. Three bands of black paper covered the areas between the 100 and 200 cc. marks, the 300 and 400 cc. marks, and an equal area above the 500 cc. mark. Thus alternating bands of light and shadow, having the same areas were produced. Of the 127 larvæ which attached to the cylinder, 82 attached in the darkened areas, five to the sides in the undarkened areas, and forty on the bottom. At another time larvæ just liberated from the parent colony were introduced into a glass cylinder of 1000 cc. capacity the sides and top of which were completely jacketed with black paper. The cylinder was held in an oblique position at an angle of less than 45 degrees, and a 75-watt nitrogen bulb was so placed that rays of light parallel with the long axis of the cylinder passed into the column of water thru the uncovered bottom. When sufficient time for metamorphosis had passed, the location of the metamorphosing zooids was noted. A very large proportion were found at the upper end of the cylinder, away from the source of light. These results indicate that the larva is not positive in its response to gravity at the end of the free-swimming period, but that its negative response to light is effective in leading it to the darkest part of the container. The lowest part of the container, and in its natural habitat, the lower levels of water are in general the darkest regions to which the larva can gain access. Hence the negative response to light performs very much the same function as would a positive response to gravity.

Other Responses.—In addition to these responses of a type commonly studied, the larvæ showed a reaction of another sort which appears to be of significance in relation to its habitat. One phase of this reaction, the response of turning abruptly upward when stimulated by a passing shadow, has been mentioned in the paper by Dr. Grave and myself. It was found on further observation that this abrupt upward turn occurred not only when the larva was stimulated by a passing shadow, but that it is a common response at other times also. As has been previously noted, the larva does not swim continually, but passes thru alternating periods of activity when it swims vigorously, and inactivity, during which it sinks, motionless thru the water. The behavior of one hundred larvæ on resuming activity after a period of quiescense was noted and recorded. Nineteen of the larvæ on resuming activity swam downward, seventeen swam in a horizontal direction, and sixty-four swam upward. These and other similar observations indicate that the larva has a strong tendency to swim upward when stimulated to renewed activity after a period of rest. What determines this response is a separate question. One stimulus, quite evidently, comes from the passing of a shadow; others, in all probability, are internal in origin.

It was noted also that larvæ frequently swim upward when they come in contact with a vertical surface. The behavior of one hundred larvæ on coming in contact with the vertical wall of the container was noted. Of these, sixteen attached themselves temporarily to the container at the point of contact; sixteen swam downward; seven swam in a horizontal direction, and sixty-one swam upward. At another time similar observations were made on the behavior of larvæ on coming in contact with a piece of black paper suspended in a perpendicular position in the aquarium. Of the hundred cases observed, two ceased activity and sank; eleven swam along the surface in a horizontal direction, and eighty-seven in an upward direction. These results suggest that the larvæ have a tendency to follow surfaces with which they come in contact in an upward direction, particularly if the surface is opaque.

Do these various reactions—the responses to light and gravity, and the "habit" of swimming upward when resuming activity or when coming in contact with a perpendicular surface—have an adaptive value to the species? When considered in relation to the natural habitat, they assume considerable significance. Adult colonies of *Botryllus* are attached very commonly to eel grass, or other objects projecting above the bottom, such as the submerged parts of floats, or rockweed. They are always found below low tide mark, and never on the bottom, being found only on the parts of eel grass blades which are not exposed at low tide, and the submerged parts of logs. Sand or mud on which the eel grass thrives offers no place for attachment and metamorphosis to the larva. It is evident from a study of the distribution of the adults that those larvæ which attach too near the surface or which sink to the bottom do not reach the adult condition. The responses of the larva are such as to keep it away from these unfavorable environments. The positive response to light and the negative response to gravity at the beginning of the free-swimming period serve to bring the larva to the surface and to distribute it more widely than would be probable without these responses. There follows a period of indifference when the larvæ show random movements; they do not orient to stimuli either of light or gravity. During this period the adaptive value of the response of swimming upward when resuming activity, or when coming in contact with a surface is evident. The negative response to light, were it unmodified by these additional responses might force the larva at the time of metamorphosis into a position too near the bottom. The response of swimming upward keeps it away from the bottom, and tends also to keep the larva in proximity to objects suitable for attachment. In experiments such as were first described, in which larvæ were allowed to metamorphose in crystallization dishes containing eel grass blades stretched obliquely from the bottom of one side to the top of the other, larvæ were many times observed to be trapped by these responses in the region of the grass blades. Larvæ sinking motionless thru the water, frequently came within range of shadows cast by the grass. Instantly they would resume activity in an upward direction.

Sometimes this resulted in bringing the larva in contact with the eel grass immediately, in which case it would follow up the surface of the blade for a few centimenters before lapsing again into inactivity. More often it failed to touch an eel grass blade, and after swimming upward a centimeter or so, it would relapse into inactivity, only to be stimulated again as it sank into the shadow a second time. The value of these responses to the larva when in its natural environment is strikingly evident.

If, as it appears, these responses function in a way which tends to lead the larva to a suitable place for metamorphosis, we have a basis for the belief that they are of survival value to the species, and that they have been in the past and perhaps are still operative in its continued evolution.

THE CHEMICAL SENSE AND FEEDING BEHAVIOR OF *NEREIS VIRENS.* SARS.

MANTON COPELAND AND H. L. WIEMAN,

Marine Biological Laboratory, Woods Hole, Mass.

In 1873 Verrill stated in his report on the invertebrates of Vineyard Sound that the clam worm, *Nereis virens,* "feeds on other worms and various kinds of marine animals. It captures its prey by suddenly thrusting out its proboscis and seizing hold with the two terminal jaws; then withdrawing the proboscis, the food is torn and masticated at leisure. . . ." Maxwell (1897) confirmed Verrill's conclusions as to the character of its food. He found that if a small piece of worm on the end of a needle is placed within reach of a normal *Nereis,* the animal seizes the food and devours it. More recently, however, Gross (1921) has failed to find any evidence that *Nereis virens* is a carnivorous worm; concluding that it feeds principally upon plant life. This verdict was based upon studies in the laboratory and in the field.

Our own observations began with dropping crushed periwinkles [*Litorina littorea* (Linn.)] in shallow water at low tide in order to study the responses of crustaceans. That such bait would cause *Nereis* to react was a thought that had not occurred to us, so that our surprise can easily be imagined when presently a worm extended the anterior end of its body from below smooth sand, moved toward the snail, seized and quickly jerked it down into its burrow. What we saw was a confirmation of Verrill's statement noted above.

Subsequent tests with the same food resulted in numerous responses of a similar nature until it appeared that *Nereis* was reacting with remarkable precision to small amounts of materials emanating from the crushed snails. A worm usually emerged several centimeters from the snail, and advanced rather slowly over the surface of the sand by movements of the body and parapodia toward the bait, which it finally seized in its powerful jaws and drew rapidly to its burrow by a sudden muscular contraction. *Nereis* responded in the same way to all other forms of animal food which were offered, viz., crushed clam [*Mya arenaria* Linn.], mussel [*Mytilus edulis* Linn.], bits of fish [*Fundulus heteroclitus* (Linn.)], and meat of the blue crab [*Callinectes sapidus* Rathbun]. When the food was grasped it was always pulled to the mouth of the burrow and, unless too large, into the interior of the burrow itself. In no case was a worm observed to leave the burrow entirely and move about freely in the water.

In order to learn something of the extent to which the animals depend upon a chemical sense in such reactions a number of tests were made, one of which was carried out in the following manner. The position of a worm was first determined by baiting with a piece of clam which was removed before the animal had a chance to seize it. Some fragments of the same food were then wrapped in cheese cloth and dropped into the water about five centimeters from the opening of the burrow. Equidistant from the burrow and one centimeter from the first packet was placed a second, consisting of a white pebble done up in cheese cloth. Each packet measured slightly over two centimeters in diameter and both had essentially the same appearance. The worm soon responded by emerging from the burrow and, advancing in a straight line toward the *baited* packet, seized it and dragged it down almost out of sight. The packet was then dug out and replaced in its former position. In a few minutes the worm reappeared and repeated the reaction in every detail. Again the food was recovered, and this time the positions of the baited and unbaited packets were interchanged. Within several minutes the anterior end of the worm came out of the sand somewhat nearer to the packets than before and moved forward in a course which, if adhered to, would have brought it between them. However, when close to the packets the animal suddenly turned and fastened its jaws in the baited one and for the third time pulled it into the sand.

The results of this experiment indicate that sight plays little or no part in the worm's food reactions under the conditions prevailing. Tests were also made by dropping pebbles near occupied burrows to ascertain if agitation of the water, or some physical factor, influenced the animal's response. To such stimuli no response occurred, although the worms appeared promptly when crab's meat was placed in the same situations. The evidence, therefore, supports the view that a chemical sense is the primary one upon which its responses to animal food depend.

Certain striking features of recorded behavior may now be considered. It was early noted that when a worm appeared from beneath the sand in response to chemical stimulation there was little uncertainty shown in the direction of its movements. Almost invariably it advanced toward the bait which, if not too far away, was generally found without difficulty. It was also observed that, when the food was out of reach, the worm withdrew into its burrow only to reappear, frequently in a position nearer the source of the stimulating material. In order to study these directive reactions further a number of animals were collected and brought into the laboratory. They were kept for a time in dishes containing water and sea lettuce, and under these conditions they would often take from forceps small pieces of meat offered them. In fact, all of the foods to which the worms reacted in their natural habitat were also accepted as they moved about in the folds of sea lettuce, enclosed more or less by mucous secretions. Under these conditions, however, they were easily disturbed and their somewhat erratic feeding behavior indicated that they were unfavorably situated for any detailed experimental study of their chemical reactions. Accordingly four worms were placed in a circular glass dish having an inside diameter of 28 cm. and containing sea water and sand. The layer of sand was approximately 2.5 cm. deep. The animals immediately entered the sand, forming burrows lined with mucus which here and there connected with the surface by well marked openings. They showed no tendency to leave the burrows if the dish remained undisturbed. After preliminary tests, which demonstrated that the worms would react to food juices as readily as they did in their natural surroundings, three similar experiments were performed, one of which is described in some detail in the following paragraphs.

August 3, 1923.

9:32:00 A. M. The water was gently stirred with a glass rod and the dish kept under observation for ten minutes. The worms remained in the sand.

9:51:00. A clam (5.4 cm. in length) cut up on half shell was placed in the center of the dish and the water stirred as before. The worms appeared as follows: worm I at 9:52:15, seven centimeters from the edge of the dish; II at 9:53:17; III at 9:54:45; IV at 9:56:50. Worms II, III, and IV appeared at the periphery of the dish.

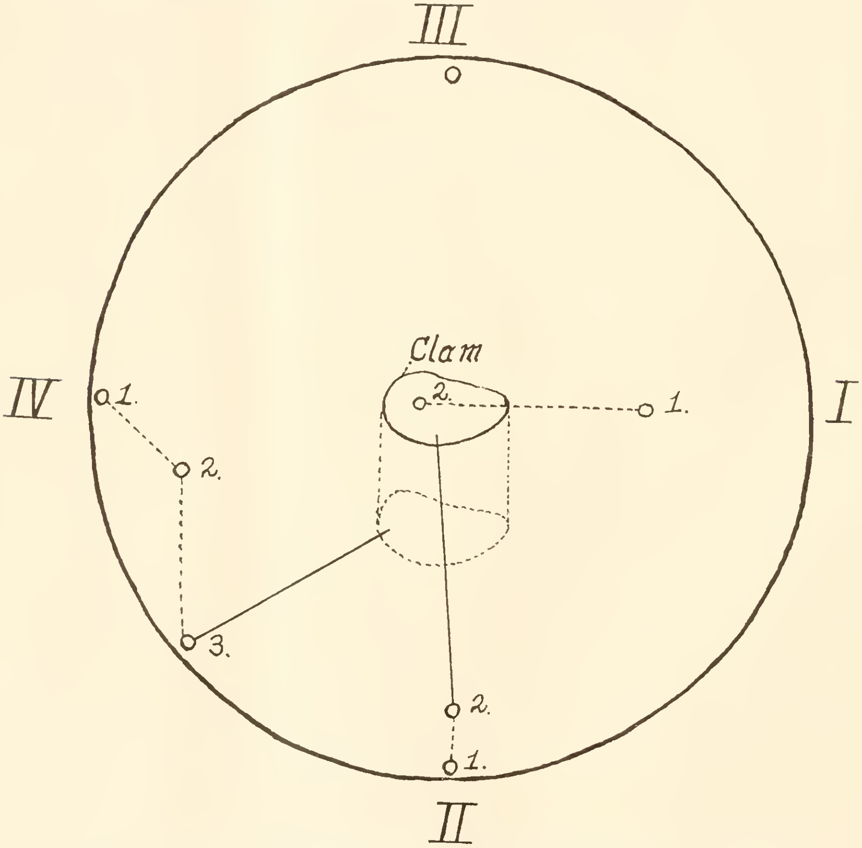

Fig. 1. Illustrating the responses of worms I., II., III., and IV., described in the text. The arabic numerals denote successive appearances of individual worms.

Worm I.—At its first appearance it made a few wavering movements with the anterior segments of its body at the mouth of the burrow and then withdrew. The same reaction was repeated a few minutes later. Finally at 10:07:30 it was found with its head exposed directly beneath the clam to which point it had burrowed, a distance of 7 cm.

Worm II.—It repeated its first appearance at the edge of the dish, withdrawing quickly each time. At its third appearance the worm proceeded directly toward the clam, exposing about 10 cm. of its body, and then quickly disappeared within its burrow. At 9:59 it came out of the sand 2 cm. nearer the clam, stretched out until it reached the clam, but finding a small fragment nearby, it seized the fragment and withdrew without disturbing the clam. At 10:07:30 the worm again emerged, this time within 2 cm. of the clam, which it grasped and dragged a distance of 3 cm., partially burying its prize in the sand. When the clam was dislodged the head of worm I. was disclosed directly beneath. At 10:13:30 worm II. reappeared at the mouth of its original burrow at the edge of the dish but made no effort to reach the clam.

Worm III.—Emerging first at the edge of the dish it immediately moved toward the clam, not quite reaching it. After withdrawing it came out again at the same point at 10:11, but by this time the clam had been pulled 3 cm. away by worm II. It drew back into its burrow and was not seen again.

Worm IV.—When first noted at the edge of the dish the worm made a few wavering movements and then disappeared, coming up again for a short time 3 cm. away. Finally at 10:17:30 it was observed at the edge of the dish, but 10 cm. from the first opening. It advanced directly toward the clam, seized it but failed to move it because of the position in which it had been wedged in the sand by worm II. After tugging at it a while, and probably biting off a piece, the animal withdrew.

The results of this experiment and two others carried out in the same way confirmed beyond question our observations made in the field. Although a worm on coming to the mouth of its burrow usually advanced the anterior end of its body in a direction toward the clam, it perhaps showed somewhat less certainty in this phase of its response than when in its natural environment. This, however, was to be expected, for in the dish well defined water currents bearing food juices, which undoubtedly aid under natural conditions in directing the animal toward the food, were largely absent, and the stimulating material must have become rather generally distributed through the water. The animals, nevertheless, after stimulation, instead of confining their activities to the periphery of the dish, near which they usually appeared, exhibited a marked tendency on failing to reach the clam to move toward the center of the dish. The explanation of how the worm maintains its direction toward the source of stimulation after withdrawing into the sand is not clear. It appears likely, however, that this directive response is a movement toward areas where the chemical substances given off by the food, and infiltrated through the sand, show higher concentration. The sense of sight plays an insignificant if any part in these reactions, for if a pipetteful of filtered clam extract is substituted for the clam in an experiment like that just described, the worms show the same responses, extending their bodies toward the center of the dish where the extract was placed.

In the laboratory as well as in natural surroundings we never saw the animals lose contact with their burrows during their movements over the sand in response to food excitation. They were tested by moonlight in the laboratory with food but no difference from behavior in daylight was noted.

For some time after the first observations on *Nereis* were made we were unable to explain how the stimulating material derived from the small quantity of food used in some of our experiments penetrated the burrows in sufficient amounts, and quickly enough, to call forth such prompt reactions. The solution of this problem was found when worms were allowed to enter glass tubes, open at both ends, the calibers of which were nearly the same as those of their burrows. Tubes of this sort are soon lined with mucus and the worms remain in them for hours at a time, exactly as they do in the sand. No difficulty was experienced in inducing them to enter the tubes. It was only necessary to direct the head into the opening, after which the worm moved forward of its own accord; and once well inside stopped locomotion. In the tube *Nereis* exhibits a most striking form of behavior which may be described as a rhythmic undulatory movement of a portion of the body taking place in a dorso-ventral direction, the parapodia remaining passive. These muscular waves were sometimes limited to the anterior end, in which case a nodding of the head occurs; at other times they appeared only at the posterior end, or perhaps in a position nearer the middle of the body. The movement is not uninterrupted but never discontinued for any length of time. This activity produces a current in the water which passes along the animal's body from the anterior to the posterior end.

That these body movements also occur when the animals are in their burrows was clearly demonstrated by two individuals kept in glass dishes containing sand. In one instance the nodding of the head mentioned above was seen through the glass as the animal lay in its burrow at the side of the dish. Carmine grains dropped in the water a few centimeters above an opening of the burrow were immediately drawn inside. In another case a long burrow was formed against the glass and here the undulatory movements of the worm were distinctly visible. From this burrow three tunnels led to openings at the surface close to the glass. From this it is clear how *Nereis,* concealed in its passageways within the sand, receives not only a constant supply of fresh water but also may be stimulated by any chemical change in the water above.

Worms occupying glass tubes respond to chemical stimulation of food as readily as those in burrows. Sea water squirted from a pipette close to a tube results in no response, but when a filtered extract of clam is introduced in the same manner the worm starts forward, moving quickly toward the end of the tube toward the juice, thrusting out its head and seizing a bit of clam dropped near the tube, or held in the forceps. This reaction was observed many times and with several individuals, and appears to be subject to remarkably little variation. By use of the glass tube every movement of the worm can be seen perfectly, and the method promises to be an excellent one for the study of the distribution of the receptors involved in the responses. Experiments designed to throw light on this aspect of the problem are already under way and will be reported upon at a later time.

SUMMARY.

1. *Nereis virens* is carnivorous, although in the absence of other food it has been observed to feed upon sea lettuce. Under natural conditions it undoubtedly is omnivorous, since Gross has found evidence of the presence of plant food in the digestive tract.

2. *Nereis* was never observed to leave its burrow when baited with meat of various marine animals, but it may expose all except the posterior segments of its body in reacting to the bait. This does not mean that the worm may not leave its burrow under other circumstances. The animal is highly thigmotactic.

3. There is positive evidence that *Nereis* depends upon a chemical sense in finding animal food; sight playing little if any part in the act. Currents in the burrow produced by an undulatory body movement are undoubtedly a factor in conveying food stimuli to the sense organs.

4. *Nereis* shows a marked tendency to extend its body from the burrow in the direction of food, and failing to reach it, to reappear in a new position nearer the source of the stimulating material.

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NEMATOCYSTS OF *MICROSTOMA.*

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For a long time the nematocysts of *Microstoma* were considered to be structures that the worm elaborated as did *Hydra.* Martin ('08) published an account of the manner in which *Hydra* is attacked by *Microstoma* and indicated his conviction that the namatocysts of *Microstoma* were derived from the Hydras that had been eaten. Kepner (11) published an account of the histological details involved in the process of transporting the nematocysts of *Hydra* from the enteron to the epidermis of *Microstoma.* In this account the inference was made that this complicated series of phenomena must mean that *Microstoma* handled these nematocysts in this manner in order to use them. This inference was early challenged by Glaser (11). A tentative reply was given Glaser's note in *Science,* Volume 34, page 213, 1911.

The details of the processes involved when *Microstoma* deals with the nematocysts are so variable and so intricate that it was felt that these processes could mean only one of two things, namely, either this was a method of eliminating indigestible foreign bodies or a method of securing weapons of defense or perhaps offense.

From time to time since 1911 efforts have been made to determine which of the two meanings was behind this conduct of *Microstoma.* This paper represents the results of our efforts that have thus extended over a decade and have been taken up each year of this decade to a certain extent.

Material and Methods.

Microstoma caudatum is found rather abundantly in the early spring months in the submerged detritus along the banks of fresh water ponds about the University of Virginia. Sometimes Hydras are found living with it in great numbers; at other times there are few Hydras. When many Hydras are present it is difficult to find Microstomas that lack nematocysts. When there are few Hydras living with *Microstoma* in nature, specimens that lack nematocysts are readily obtained.

It has been found that by placing *Microstoma* in watch-glasses containing fresh water and some fragments of water soaked, dead leaves they can be kept indefinitely; provided the air be kept free of coal gas, illuminating gases, and formaldehyde vapor.

Nematocysts Passing from Endoderm into Mesoderm.

While this paper is not dealing with the methods by which *Microstoma* handles nematocysts, it is of interest to record that Kepner and Whitlock in 1917 observed a nematocyst being carried down through the body of an endodermal cell and delivered through the basement membrane into the mesenchyme. This was a very slow process. The movement of the nematocyst could be appreciated only when its position was recorded and then five minutes later its position again observed. Its rate was not accelerated as it passed through the basement membrane into the mesoderm. The most remarkable feature of this was that the obtuse end was directed towards the path along which the nematocyst was being transported.

Nematocysts Uniformly Distributed.

Attention might be called to the additional fact that when these nematocysts are taken up by the mesenchymal cnidophages they are distributed uniformly at the epidermis by these attending cells. So that immediately after a *Microstoma* has acquired a supply of nematocysts at its surface, these nematocysts are uniformly distributed. It does not appear, however, that if the anterior end, or any other region, lose its quota of nematocysts that some will be taken from other regions of the body to take the place of those lost. In this manner specimens sometimes come to have a uniform distribution of nematocysts except for some region (e. g. "head") that is free from them. Despite this fact, the subtle manner in which the cnidophages coöperate to bring about a uniform distribution of the foreign nematocysts is remarkable and lends weight to the inference that the nematocysts are being collected by the *Microstoma* to be used.

Nematocysts Retained Indefinitely.

Again if the nematocysts of *Hydra* are carried out to the surface of *Microstoma* by way of eliminating them as indigestible foreign bodies, the question arises as to why they are held so long at the surface. Within twelve hours after a *Hydra* has been ingested by *Microstoma,* the nematocysts are distributed over the rhabdocoele's body. The process of getting these objects to the surface of the flatworm is, therefore, a matter of about twelve hours. When, however, they arrive at the surface they are held there indefinitely. Even when animals are kept under adverse conditions in the laboratory and they show more and more conspicuous decline, the nematocysts are yet retained. Specimens have been seen growing weak and beginning to rupture or break up and yet retain the nematocysts over the epidermis that remained intact. This retention of the nematocysts, likewise, suggests that they have been collected for use.

An Expensive Method.

Again this is an expensive method of dealing with objects that could be thrown out at the mouth as are other indigestible materials. For in this process much energy must be spent by the endodermal cells and cnidophages in handling the nematocysts; and, further, when the nematocysts are discharged they carry with them their attending cnidophages. The cnidophages, therefore, in attending the nematocysts act against their own welfare in that in the end they lose their own lives.

Microstoma Incurs a Danger.

Even the *Microstoma* as a whole feeds upon *Hydra* at a risk. For sometimes *Hydra* turns upon *Microstoma* and eats it. *Hydra,* however, seems to have difficulty in ingesting *Microstoma* as the following observation of January 18, 1917, shows. The *Microstoma* at once played along the side of *Hydra viridis* that had been placed with it. The worm passed to and fro along the surface of the polyp's body. Twice it came to rest amidst the bases of the tentacles. The third time it came to lie over the mouth of the *Hydra,* the latter began an effort to ingest the *Microstoma* (Fig. 1). First the *Hydra's* peristome opened up against the ventral side. This region of the peristome did not fix the *Hydra* to the *Microstoma,* for the mouth of *Hydra* glided posteriory along the ventral side of *Microstoma* and around its posterior end as indicated by the arrow in Fig. 2. When the widely expanded peristome of *Hydra* had come to be applied over a great part of the dorsal and lateral surfaces of the *Microstoma,* the *Hydra* pressed the latter between its peristomal bell and its body and appeared to have the worm in a very serious position (Fig. 3). But from this embrace the *Microstoma* soon glided. As it escaped it showed a ruptured region of its body. This wound healed in a little while and then *Microstoma* went back and played along the surfaces of *Hydra's* body. The next time it came into the tentacular zone of *Hydra,* the polyp succeeded in grasping the *Microstoma* head-on and forthwith ingested it (Fig. 4). Since, therefore, *Microstoma* incurs a greater danger in seeking out *Hydra* than it does when it feeds upon small annelids, crustacea and other small animals and plants and since, further, it does not appear to seek *Hydra* primarily for food as will be shown later, the inference is strong that it is seeking *Hydra* for some unique end.

Microstoma Able to Draw Nematocysts from its Body when Wounded by *Hydra.*

Moreover, *Microstoma* seems to have developed an adaptive secretion by its epidermal glands that may be taken to be either rhabditic glands or the homologues of such glands which are found in other rhabdocoeles. The following observations indicate the adaptive functioning of these glands. February 15, 1917, a *Microstoma* was placed with a small *Hydra* fusca. The *Microstoma* began playing about the five tentacles. Immediately the *Hydra* firmly grasped it between its tentacles (Fig. 5). The *Microstoma* escaped from the *Hydra's* embrace. As it swam clear of the polyp, two mucous masses were to be seen, one at the posterior end and one on the right side. (Fig. 6). The lateral mass grew and as it grew drew the two enclosed nematocysts out from their anchorage within the worm's body and then glided along the right side of the body posteriorly to where it fused with the posterior mass, which had three nematocysts enclosed within it. This compound mass of mucus, now containing five freed nematocysts, was sloughed off from the *Microstoma's* body and left behind. After that the *Microstoma* seemed to be enveloped in a transparent mucus sheath to which small dead objects adhered and were dragged about with the *Microstoma* until they became entangled with the tentacles of the *Hydra*. These tentacles stripped the foreign bodies from the *Microstoma's* adhesive surface. Another observation of this sort was made January 29, 1917. A *Microstoma* was placed with a large budding *Hydra fusca*. It at once attacked the *Hydra*. The *Hydra* discharged a nematocyst into the anterior end of *Microstoma*. The *Microstoma* contracted so vigorously as to break apart the two nearly formed zooids that had been developing through fission. About the deeply anchored nematocyst the anterior zooid now secreted a local mass of mucus. This mass of mucus grew until it formed a column whose length was more than two-thirds that of the zooid. Next the posterior zooid encountered the bud of the *Hydra*. As it passed the *Hydra*, the latter stung it in its posterior end along the left side. About this nematocyst a local secretion of mucus appeared and as the mass of mucus grew in length the long stinging thread of the nematocyst was dragged from the *Microstoma's* body as had a mucous column dragged the nematocyst from the body of the anterior zooid. Eventually the mucous masses were discarded and the two zooids appeared to be no worse for their experience.

All the above shows an intimate relation existing between *Microstoma* and *Hydra* which is peculiar and must have some meaning while much of the above indicates that the meaning of this conduct centers about the nematocysts of *Hydra*.

Microstoma WITH FEW NEMATOCYSTS REACTS TO *Hydra* READILY.

Moreover, a *Microstoma* that has few or no nematocysts behaves differently towards *Hydra* than does one that has a complete quota of nematocysts at its surface. On December 8, 1916, a *Microstoma,* that contained seven or eight nematocysts and so greatly filled with food that the pharynx of its posterior zooid was everted, was placed with a *Hydra.* Within 12 minutes the *Microstoma* had egested some of its food and had torn off a part of the *Hydra's* oral end. Two days later a second well-fed specimen, that contained but two nematocysts, was placed with a *Hydra.* This specimen ate the *Hydra* within twenty minutes. Table I., shows that of 42 specimens containing none or few nematocysts, 6 (within two minutes after being placed with Hydras) set to work trying to feed upon the polyps, but five of them got severely wounded while the sixth was eaten by the *Hydra.* Two others were eaten by the *Hydra* within twenty-four hours. Of the remaining 34 specimens, 9 had eaten Hydras in periods ranging from 2 minutes to 20 minutes; while 20 accepted Hydras in periods ranging from 24 minutes hours to 1 hour; and 3 remained with *Hydra* either 5 or 2 days before they accepted *Hydra.* Two specimens remained with *Hydra* 24 hours and a third 4 days when they were lost or had died without having accepted *Hydra.* The conspicuous feature of Table I. is that in many cases *Hydra* was accepted within a period of minutes, many others within a period of hours, while only a few were accepted within a period of days.

Microstoma WITH MANY NEMATOCYSTS REACTS TO *Hydra* TARDILY.

Table II. makes a sharp contrast with Table I. in this respect. For this table shows the reactions of 18 Microstomas, that had either many or a full quota of nematocysts. All, except specimen 15, were kept from food for a day or more and yet only one of them, specimen 7, reacted to *Hydra* within 24 hours and this one contained only about one-third of a full quota of nematocysts. Specimen 8 remained with *Hydra* 24 hours when it was lost, without having accepted *Hydra.* Except for these two specimens, all the others reacted to *Hydra* not within periods of hours or minutes but of days and that despite the fact that they had no food for at least a day. Specimens 17 and 18 were kept away from food in each case four days. They lived four

TABLE I.

NEMATOCYSTS OF *Microstoma* NONE OR FEW.

Specimen.	Number of Nematocysts.	Time with *Hydra.*	*Hydra* Eaten.	*Hydra* Not Eaten.	*Microstoma* Eaten.	*Microstoma* Stung.
11–21–16	None	24 hrs.	+			
12– 6–16	7 or 8	12 min.	+ though the specimen had much food.			
12–10–16	2	20 min.	+ though well fed.		−	−
12–11–16	6	3 min.	+	−	−	−
1–10–17	None	24 hrs.	+	−	−	−
1–18–17	2	2 min.	−	−	+	−
1–20–17	6–10	2 days	+	−	−	−
1–23–17	8	5 days	+	−	−	−
1–24–17	5	2 min.	−	−	−	+
1–26–17	1 or 2	2 min.	−	−	−	+
2–12–17	1	2 min.	−	−	−	+
4–19–17	10	2 min.	−	−	−	+[1]
7–23–17	Few	2 min.	−	+	−	+
7–24–17*a*	6	10 min.	+	−	−	−
7–24–17*b*	None	10 min.	+	−	−	−
8–18–17*b*	None	5 min.	+	−	−	−
9–18–17*d*	1	24 hrs.	+	−	−	−
9–18–17*e*	Few	24 hrs.	−	+	+	−
9–18–17*f*	2	24 hrs.	+	−	−	−
9–18–17*g*	7	24 hrs.	+	−	−	−
9–18–17*h*	Few	5 min.	+	−	−	−
9–19–17*a*	Few	24 hrs.	+	−	−	−
9–19–17*b*	1	24 hrs.	+	−	−	−
9–19–17*c*	None	24 hrs.	+	−	−	−
9–19–17*d*	5	24 hrs.	+	−	−	−
9–19–17*e*	5	24 hrs.	+	−	−	−
9–19–17*f*	None	1 hr.	+	−	−	−
9–19–17*g*	7	1 hr.	+	−	−	−
9–19–17*h*	None	3 min.	+	−	−	−
10– 5–17*a*	None	6 hrs.	+	−	−	−
10–17–17*a*	1	24 hrs.	+	−	−	−
10–17–17*c*	6–8	24 hrs.	+	−	−	−
10–17–17*e*	None	24 hrs.	−	+	−	−
10–17–17*f*	None	24 hrs.	+	−	−	−
10–17–17*h*	8–10	24 hrs.	+	−	−	−
10–17–17*j*	None	24 hrs.	−	+	−	−
10–17–17*l*	Few	24 hrs.	−	+	−	−
10–17–17*m*	Few	24 hrs.		−	+	−
9–18–17*e*	Few	2 days	+	−	−	−
5– 3–23	Few	4 days	−	+	−	−
5– 3–23	10	24 hrs.	+	−	−	−
5– 8–23	None	24 hrs.	+	−	−	−

days with *Hydra* before they died without accepting any part of *Hydra.* Specimen 16 is the most significant one of the table. It was learned by observation that *Microstoma* could live in a small vessel of spring water for about nine days without food. Many died in less time under these conditions but none passed

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[1] Rather by gastric juices than by nematocysts.

TABLE II.

NEMATOCYSTS OF *Microstoma* MANY.

Specimen.	Loaded with Nematocysts.	Without Food.	With *Hydra.*	*Hydra* Eaten.	*Hydra* Not Eaten.	Nematocysts Thrown from Enteron of *Microstoma.*
(1) 9–18–17*B*	1/2	1 day	2 days	+	–	–
(2) 9–18–17*D*	1/2	"	"	–	+	Sick; see notes.
(3) 9–18–17*F*	4/5	"	"	+	–	–
(4) 9–18–17*G*	1/2	"	"	+	–	–
(5) 9–19–17*B*	1/3	"	"	+	–	–
(6) 9–19–17*C*	1/2	"	3 days	+	–	–
(7) 9–19–17*D*	1/3	"	1 day	+	–	–
(8) 9–19–17*D*	Loaded	"	"	–	+	–
(9) 9–19–17*E*	2/3	"	3 days	+	–	–
(10) 9–19–17*F*	1/4	"	"	–	+	– Sick; see notes.
(11) 9–19–17*G*	1/4	"	"	+	–	–
(12) 9–19–17*H*	Loaded	"	"	–	+	
(13) 9–19–17*H*	4/5	"	2 days	+	–	+
(14) 9–19–17*A*	1/2	"	"	+	–	–
(15) 9–19–17*A*	Loaded	No[1]	"	+	–	+
(16) 11–21–16	Loaded	9 days	"	–	+	Piece of tadpole liver accepted immediately after the *Microstoma* was taken from the *Hydra* of specimen (16).
(17) 5– 6–23	1/2	4 days	4 days	–	+	
(18) 5–10–23	1/5	"	"	–	+	

the twelfth day. With this fact in mind a "loaded" specimen was kept away from food for nine days. It was placed with *Hydra* at the end of the ninth day and remained with it for two more days without accepting *Hydra.* This was not due to the *Microstoma's* condition having been so greatly lowered that it could not accept food. For when it was taken from *Hydra* and given a piece of tadpole liver, the *Microstoma* immediately accepted it. The facts tabulated in these two tables indicate that a *Microstoma* with few or no nematocysts attacks a *Hydra* much more readily than does one with many or a full quota.

Microstoma MAY EGEST CELLS OF *Hydra* AND RETAIN NEMATOCYSTS.

Another contrast may be drawn between the conduct of a *Microstoma* that has few or no nematocysts and that of one that has a full quota of nematocysts. If a *Microstoma* that contains a *Hydra,* so long ingested that the polyp has been reduced to a pulp, be placed under slight pressure it will discharge the green protoplasmic mass of the *Hydra's* body and retain the nematocysts within its enteron. This does not appear to be due to the fact that the pressure has held fast the mass of nematocysts; for, during the process of egesting the other *Hydra* material, the nematocysts are being thrust to and fro within the enteron. Under such condition a *Microstoma,* therefore, rejects the food and retains the nematocysts.

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[1] Not starved since it had eaten *Hydia* as specimen 14.

Microstoma MAY EGEST NEMATOCYSTS AND RETAIN CELLS OF *Hydra.*

The senior author saw just the reverse of this. A *Microstoma* was loaded with nematocysts by being fed a *Hydra.* It was then starved until it would accept *Hydra.* While the *Microstoma* was kept under observation the *Hydra* was digested. When alimentation had been completed the indigestible nematocysts were thrown out of the mouth and rejected. When Dr. W. H. Taliaferro, now of Johns Hopkins University, was told this he expressed skepticism; his challenge was accepted by the senior author to have this demonstrated. From 10.58 A.M. to 11.14 A.M., September 19, 1917, Dr. Taliaferro kept the "loaded" *Microstoma* under observation. During this time he could see the nematocysts of a recently ingested *Hydra* within the enteron. At 11.14 A. M., he saw the nematocysts being discharged from the mouth of the "loaded" *Microstoma.*

From these observations it appears, therefore, that *Hydra* is eaten by *Microstoma* not primarily as a food but for its nematocysts. Thus, it is further suggested that the handling of the nematocysts by *Microstoma* is done in order that the rhabdocoele may use these "stinging threads."

DISCHARGE OF NEMATOCYSTS BY MICROSTOMA A DOUBLE PROCESS.

When one teases a *Microstoma,* that is armed with *Hydra's* nematocyst, the nematocyst in the immediate neighborhood of the stimulus oscillates to and fro at right angles to the surface of the body. If the teasing is maintained long enough, the nematocyst will be discharged at the object with which the *Microstoma* was being touched. An observer cannot watch this double reaction on the part of the *Microstoma* when being stroked with a needle point without feeling that the first phase of reaction, viz., the oscillation of the nematocyst, is a threat and that the second phase of the reaction, viz., the discharge of the nematocyst, was an effort to use the nematocyst against the annoying object.

Microstoma Uses Its Nematocysts.

Finally we have been able to see *Microstoma* actually using the nematocysts that it had appropriated from *Hydra*.

October 13, 1917, the senior author placed some Stenostomas in a hanging drop with a *Microstoma* that had nematocysts. One of the Stenostomas was at once stung along its side. The wound caused the Stenostoma's body to rupture. After the *Microstoma* had thus wounded the victim, it at once swallowed it. Mr. Conway Zirkle saw this same *Microstoma* strike another *Stenostoma* in such fashion as to cause it to bend its body near the middle at right angles and to remain quiet in this contour until the *Microstoma* fell to ingesting it. In neither of these observations were the actual nematocysts seen entering or having entered the *Stenostoma*.

But on September 9, 1917, the senior author placed a large *Microstoma*, containing nematocysts, in a hanging drop of water with three very large dividing Stenostomas. The head of one of these Stenostomas came in contact with the right side of the *Microstoma's* head. Forthwith the *Stenostoma* contracted violently. From a wound at the tip of the body the mesenchyme oozed. As this material ran out of the body, it dragged with it two nematocysts that had their filaments and barbs ejected. The *Stenostoma's* body also developed a small blister to the left of the ruptured region of the epidermis. The *Microstoma* left the *Stenostoma*, which no longer moved from place to place, and later came back and attempted to ingest the *Stenostoma* as it lay struggling as though suffering from its wound.

September 19, 1917, the senior author placed a *Microstoma*, that had nematocysts, in a hanging drop of water with a single dividing *Stenostoma*. Eight times these two animals collided.

Sometimes these collisions caused the one animal to glide down along the side of the other; at other times the collisions would be head on. In each case the collisions were so evident as to cause a shunting of one or the other specimen. At 11.30 A.M. while *Microstoma* lay quiet, *Stenostoma* made a contact with *Microstoma* and stroked the entire left side of the *Microstoma* with its right ciliated pit. When *Stenostoma's* head had come to be in contact with the posterior end of *Microstoma,* the latter discharged two nematocysts into the region of *Stenostoma's* right ciliated pit. At once a rupture took place in the *Stenostoma's* epidermis and from this wound granular material oozed. Despite this breaking of the *Stenostoma's* body in the region in which it had been stung, the *Stenostoma* was anchored to *Microstoma* by the nematocysts, the poison sacs of which were yet held within the *Microstoma.* The two animals were anchored thus long enough for me to call Dr. I. F. Lewis to my side and make a demonstration of the situation to him. Soon after this demonstration was made, the two poison sacs left the *Microstoma's* body and the latter swam away. The *Stenostoma,* which up to the time it was stung had been incessantly active, lay quite inactive for ten minutes with two nematocysts hanging from the wound on the right side of its anterior end. After that it gradually recovered itself.

It has thus been demonstrated that *Microstoma* uses the nematocysts which it takes from the *Hydra.*

Summary.

1. *Microstoma* manipulates the nematocysts of *Hydra* either (*a*) as a means of eliminating indigestible parts of its food, or (*b*) a means of defending itself.

2. The fact that the nematocysts are distributed uniformly over the surface of *Microstoma* within 12 hours after a *Hydra* has been ingested, suggests that this is not a process of elimination, (*a*), but is done in order that the nematocysts may be used, (*b*).

3. The fact that the nematocysts are retained indefinitely suggests that they have been taken up and retained for use.

4. The fact that this method of handling the nematocysts demands considerable work on the part of the endodermal cells and, in each case of a discharged nematocyst, the death of a cnidophage or mesodermal cell further suggests that it is all done with reference to use.

5. *Microstoma* incurs danger in attacking *Hydra;* for it is frequently stung and sometimes killed by the polyp. This too suggests that the *Microstoma* attacks *Hydra* for some peculiar end.

6. The *Microstoma* is able to draw nematocysts with which *Hydra* has stung it from its body, without suffering a rupture as does *Stenostoma.*

7. *Microstoma* with a few nematocysts attacks *Hydra* within a little time.

8. *Microstoma* having many nematocysts does not readily attack *Hydra.* The contrast between 7 and 8 suggests that *Microstoma* attacks *Hydra* not for food; but for its nematocysts.

9. The alimentary canal of a recently fed *Microstoma,* that has few nematocysts, may egest the "flesh" of *Hydra* and retain its nematocysts.

10. The alimentary canal of a *Microstoma* that has many nematocysts and is hungry, may egest the nematocysts of *Hydra* and retain the "flesh" of the polyp. The contrast between 9 and 10 suggests that *Microstoma* seeks primarily the nematocysts of *Hydra.*

11. The discharge of the nematocysts is a double process, involving (1) an oscillation to and fro of the nematocyst within its cnidophage, and (2) the actual discharge. The first phase may be carried on without being followed by the second phase. Neither of these has ever been seen except when some active body, like the experimenter's needle or an animal has brushed along the surface of *Microstoma.* This, too, suggests that the nematocysts are for use.

12. Finally *Microstoma* actually stings and paralyses other animals with the nematocysts it has appropriated from Hydras that it has eaten.

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EXPLANATION OF FIGURES.

PLATE I.

FIG. 1. A *Microstoma* slowly moving amid the bases of the tentacles of *Hydra*. × 50.

FIG. 2. In response to the presence of *Microstoma* the *Hydra* has spread its expanding peristome along ventral surface of *Microstoma*. × 50.

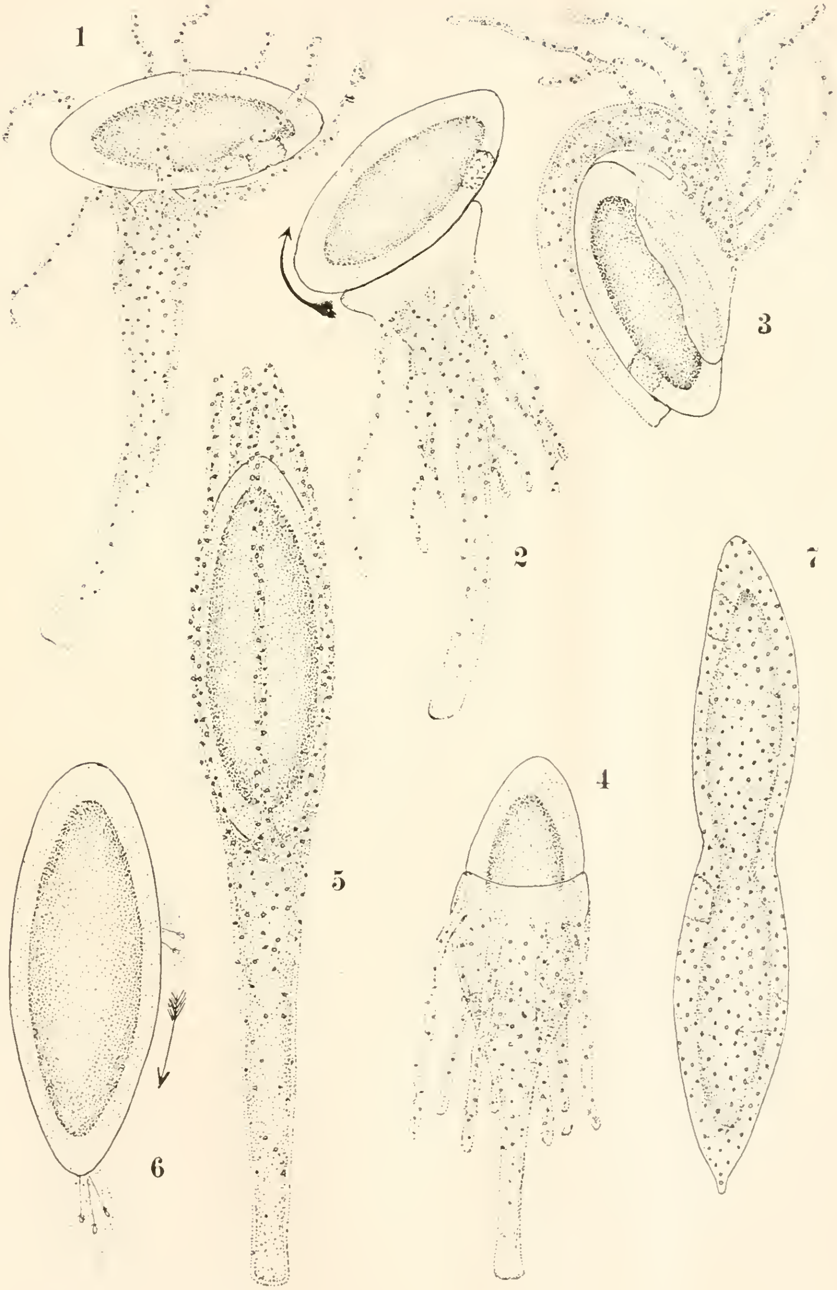
FIG. 3. The expanding peristome has glided posteriorily and dorsally, in direction indicated by arrow in Fig. 2, over surface of *Microstoma* as the *Hydra* flexed its body and pressed the *Microstoma* between its greatly expanded peristome and its bent body. From this embrace the *Microstoma* escaped. × 50.

FIG. 4. Soon after this escape the *Microstoma* returned to be captured by the tentacles of *Hydra*. After the inception of ingestion the tentacles of *Hydra* were bent back so as to lie almost parallel to the axis of the polyp. The ingestion was a slow process. × 50.

FIG. 5. Shows the manner in which a *Microstoma* was held by a *Hydra*. The *Microstoma* slowly glided out of this embrace between the ends of the tentacles. × 50.

FIG. 6. When the *Microstoma* had escaped it showed two wounded regions —one bearing two nematocysts and the other three nematocysts. These nematocysts were at first embedded so that only their poison-sacs projected from the surface of the *Microstoma*. Very early a mass of mucus formed in each wounded region. The lateral mass moved posteriorly (as shown by arrow) and fused with the posterior one. As these masses of mucus grew in length, they dragged the five stinging threads from the body of the *Microstoma*. The combined mass of mucus, containing five nematocysts, eventually was cast off. After this the *Microstoma* moved about normally. × 50.

FIG. 7. Shows a dividing *Microstoma* with a maximum charge of nematocysts at its surface. When the nematocysts are this frequent the *Microstoma* is said to be "loaded." × 50.



WM. A. KEPNER AND JOHN F. BARKER.

SOME EFFECTS OF THE LOWER ALCOHOLS ON *PARAMECIUM*.[1]

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General—Investigators of the protozoa do not agree as to the influence of abnormal environment. For example, Matheny (1910) states that alcohol in doses of two per cent. or less "has no effect whatever" on *Paramecium,* while Calkins and Lieb (1902), and Woodruff (1908), working with doses many times more dilute report marked, but dissimilar effects.

In the present studies considerable variation in the deportment of individual paramecia from a given clone was noted, which indicates that some of the factors of error in the quantitative study of *Paramecium* are obscure, and not easy of control. As Towle (1904) observes, "The sensitiveness of paramecia for different substances varies without apparent regularity." Nevertheless it was found possible in the following experiments to obtain results of significance by counting great numbers of organisms, observing strict chemical cleanliness, and confining most of the experiments to dormant cultures of pure stocks.

Cultures—Pure lines of *Paramecium caudatum* and *Paramecium aurelia* were cultivated in battery jar infusions consisting of about 25 grams of timothy hay per liter of spring water. These were twice boiled to insure the destruction of rotifers. After about a month from the date of preparation the cultures entered upon a prolonged stage of dormancy during which little detectable change occurred until starvation was evidenced by an abrupt decline. Except where otherwise noted, only organisms from the dormant cultures were studied.

No attempt was made at bacterial control. However, in one culture a mixture of *B. lactis aerogenes* and a bacillus of the

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[1] Abbreviated excerpts from an essay presented to The Johns Hopkins University in conformity with the requirements for the degree of Master of Arts (Bills, 1923*a*). A previous publication (Bills, 1923*b*) containing other excerpts should be consulted.

aquatilis group gained a long-enduring ascendency over all other bacterial forms.[2] As this culture supported the finest growth of paramecia that I have ever seen, it is interesting to note that Hargitt and Fray (1917) and Phillips (1922) maintain that simple bacterial mixtures do *not* provide as good a food for *Paramecium* as the usual complex natural mixtures.

Alcohols—The six simplest monatomic alcohols were employed: Methyl, ethyl, *n*-propyl, *i*-propyl, *n*-butyl, and *i*-butyl. All were of good purity. Dilutions in spring water were prepared volumetrically, with a micro-burette, a fresh solution being employed for each observation.

The Effect of Lethal Concentrations of the Alcohols—One clone of *P. aurelia* and two of *P. caudatum* were treated with such strengths of the four normal alcohols as sufficed to kill them in from 30 seconds to 30 minutes. The strongest concentration used was 15 per cent methyl alcohol; the weakest was 0.8 per cent butyl. All other strengths were intermediate. In about 300 individuals the process of dying was observed under a magnification of 700 diameters.

Wide variations disregarded, the phenomena usually observed follow in order: Incoördination and inactivation of body cilia; discharge of trichocysts; arrest of contractile vacuoles; modification of cyclosis in course and diminution in rate; bending of body to a crescent; convulsive rearrangement in posterior part, producing "Indian club" shape; arrest of undulating membrane in gullet; and at death, change in appearance of protoplasm to opaque and yellowish, cessation of Brownian movement, occasional formation of blisters by elevation of cuticle, and sometimes rupture of ectoplasm with discharge of endoplasm into the blisters.

Three points in blister formation warrant further mention: (1) The lower alcohols give rise to a *few* blisters which grow *rapidly*, whereas the higher ones result in *many* blisters which grow *slowly*.

(2) The existence of "susceptibility gradients" in *Paramecium* is nicely demonstrated in blister formation. Blisters

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I am much indebted to Dr. Percy D. Meader, of the School of Hygiene and Public Health, for the bacteriological examination of this unusual culture.

rarely, if ever, form in the oral groove, and this region is generally the last to exhibit the signs of death. But the anterior end is the most susceptible to blister formation, and it is there that the ectoplasm most frequently breaks. Furthermore, the aboral side is more susceptible than the oral, being nearly as delicate as the anterior end, in regard to both blister formation and ectoplasmic rupture. In this connection it is interesting to recall the work of Child (1914) who demonstrated in *Paramecium* anterior hypersensitiveness to cyanide.

(3) When the granules of the seemingly still living protoplasm are discharged thru the ectoplasm into a blister they do not behave precisely like free particles in a liquid; they keep together in globular masses, or in thread-like protrusions. Sometimes they may become differentiated even more distinctly from the still hyaline portion of the blister by forming a new superficial film. These observations are in accord with the researches of Seifriz (1921), who noted the tendency of living protoplasm to remain immiscible with water, and "to form, almost instantly, a membrane on its surface."

Alcohol and Resistance to Starvation—None of the earlier studies on the influence of alcohol on *Paramecium* appears to have considered the effect on starving cultures. In attacking this problem cultures were prepared by adding one volume of dormant stock culture to one volume of an alcohol of twice the desired strength. Such mixtures were apportioned in 25 cc. fractions to about 100 Stender dishes of 30 cc. capacity. Most of the dishes were kept at room temperature, and the covers removed only when observations were made at various intervals. A few of the cultures were temperature-controlled.

By a method described at length in my original essay (Bills, 1923*a*) determinations were made on the maintenance of the alcoholic content of these cultures. It was found that in spite of the closely fitting covers on the dishes the alcoholic content diminished at the rate of 21 per cent. of the original amount in five days, and 56 per cent. in 31 days, these values including loss by consumption as well as loss by evaporation. Both values are averages of 45 cultures containing 1.25 per cent. ethyl alcohol.

Three sets of observations were made. The results are recorded in Tables I., II., and III. Table I. is a record of the activity, size, and endurance of organisms from *Paramecium caudatum,* Clone 10 exposed for varying lengths of time to the first four normal primary alcohols in the following concentrations: 1.0 per cent. methyl; 0.8 per cent. ethyl; 0.4 per cent. propyl; 0.2 per cent. butyl. Table II. is a similar record of five discrete experiments on Clone 10 showing the difference between alchoholized and normal cultures. Controls consisting of culture diluted with an equal volume of spring water were used in this series. All the treated cultures contained 1.0 per cent. methyl alcohol. Table III. is a comprehensive record of seven cultures of paramecia,

TABLE I.

A RECORD OF THE ACTIVITY, SIZE, AND POPULATION OF CULTURES FROM CLONE 10, UNDER THE INFLUENCE OF 1.0 PER CENT. METHYL, 0.8 PER CENT. ETHYL, 0.4 PER CENT. PROPYL, AND 0.2 PER CENT. BUTYL ALCOHOLS.

Alcohol.	Exposure.	Activity.	Size.	Population.
Methyl . .	24 Hours	Normal	Decreased	
Ethyl. . . .		Increased	Normal	
Propyl. . .		Normal	Normal	
Butyl. . . .		Increased	Normal	
Methyl . .	64 Hours	Normal	Normal	Distributed throughout culture
Ethyl. . . .		Normal	Normal	Distributed throughout culture
Propyl. . .		Normal	Normal	Aggregated in dense masses
Butyl. . . .		Normal	Normal	Distributed throughout culture
Methyl . .	91 Hours	Decreased	Decreased	
Ethyl. . . .		Normal	Normal	
Propyl. . .		Decreased	Decreased	
Butyl. . . .		Normal	Normal	
Methyl . .	25 Days		Decreased	Very few
Ethyl. . . .			Normal	Few
Propyl. . .			Normal	Extremely numerous
Butyl. . . .			Normal	Extremely numerous
Methyl . .	53 Days	Decreased		Few
Ethyl. . . .		Decreased		Very numerous
Propyl. . .		Decreased		Numerous
Butyl. . . .		Decreased		Numerous
Methyl . .	120 Days			Extinct
Ethyl. . . .				Very few
Propyl. . .				Extinct
Butyl. . . .				Extinct

each of which was treated with six different alcohols, and observed as to population in comparison with untreated, undiluted, controls at intervals up to two months from the time of preparation. The concentrations of methyl and *n*-propyl alcohols seem to have been a little high for some of the cultures. The alcoholic content was as follows: 2 per cent. methyl; 1 per cent. ethyl; 1/2 per cent. *n*-propyl; 1/4 per cent. *n*-butyl; 3/4 per cent. *i*-propyl; 3/8 per cent. *i*-butyl.

Table II.

Records of Five Discrete Experiments on Clone 10 in 1.0 per cent. Methyl Alcohol, Showing the Differences Between Alcoholized and Control Cultures after Different Periods of Time.

1. Time, 16 days. Temperature maintained at 27.5°.
    Treated culture flourishing.
    Control beginning to starve.
2. Time, 30 days. Room temperature. Covers sealed with vaseline.
    Treated culture contains many large, slow-moving animals.
    Control died of starvation.
3. Time, 30 days. Temperature maintained at 25°.
    Treated culture contains many paramecia of almost normal size, but much vacuolated and very slow-moving.
    Control died of starvation.
4. Time, 30 days. Temperature maintained at 35°.
    Treated culture contains many small, active paramecia.
    Control died of starvation.
5. Time, 50 days. Room temperature.
    Treated culture contains many large, active, slightly vacuolated paramecia.
    Control died of starvation.

Inspection of the tables reveals that all alcohols have a similar influence on starving cultures. Not only do all of them postpone the advent of starvation, but they may even restore severely starved cultures to their former prosperity. This fact should not be taken to indicate that alcohols function *directly* as food for *Paramecium*, as they appear to do for green algæ (Moore and Webster, 1920). In the present case their mode of action is obscure. In activity the alcoholized paramecia remain normal, increase, or decrease; and in size they remain normal, or decrease—conditions attributable quite as well to nutritional as to pharmacological influence.

TABLE III.

A POPULATION RECORD OF SEVEN CULTURES EXPOSED, WITH CONTROLS, TO SIX ALCOHOLS FOR DIFFERENT PERIODS OF TIME.

The alcoholic strengths were: 2 per cent. methyl; 1 per cent. ethyl; ½ per cent. *n*-propyl; ¼ per cent. *n*-butyl; ¾ per cent. *i*-propyl; ⅜ per cent. *i*-butyl.

	Alcohol.	Population at Time of Prep.	Population 4 Days Later.	Population 30 Days Later.	Population 60 Days Later.
P. caudatum Clone 10	Control	Pale, odorless, nearly extinct from starvation.	Nearly extinct	Nearly extinct	Extinct
	Methyl		Extinct	....	....
	Ethyl		Multiplying	Very numerous	Excellent
	n-Propyl		Extinct	....	....
	n-Butyl		Slight increase	Very numerous	Numerous thin
	i-Propyl		Nearly extinct	Numerous	Extinct
	i-Butyl		Extinct	....	....
P. caudatum Clone 10	Control	Pale, putrid, extremely numerous. Some aggregates of paramecia have been broken up.	Excellent	Very numerous	Very few thin
	Methyl		Excellent thin	Numerous fat	Extinct
	Ethyl		Excellent	Excellent	Numerous small
	n-Propyl		Extinct	....	....
	n-Butyl		Excellent	Very numerous	Numerous
	i-Propyl		Numerous	Numerous	Extinct
	i-Butyl		Excellent	Numerous	Few but good
P. caudatum Clone 8	Control	Pale, odorless, dormant. Will soon be starving.	Excellent	Very few	Extinct
	Methyl		Nearly extinct	Extinct	....
	Ethyl		Excellent	Very few	Very few
	n-Propyl		Extinct	....	....
	n-Butyl		Excellent	Few	Extinct
	i-Propyl		Excellent	Few	Extinct
	i-Butyl		Excellent	Very numerous	Nearly extinct
P. aurelia Clone 11	Control	Colorless, odorless, few paramecia in starvation.	Excellent	Few	Extinct
	Methyl		Few	Very few	Numerous good
	Ethyl		Excellent	Nearly extinct	Extinct
	n-Propyl		Extinct	....	....
	n-Butyl		Numerous	Nearly extinct	Extinct
	i-Propyl		Excellent	Extinct	....
	i-Butyl		Very few	Extinct	....
P. aurelia Clone 7	Control	Pale, odorless, dormant.	Numerous	Nearly extinct	Extinct
	Methyl		Few	Extinct	....
	Ethyl		Numerous	Numerous	Accident
	n-Propyl		Extinct	....	....
	n-Butyl		Multiplying	Very numerous	Very few
	i-Propyl		Numerous	Few	Extinct
	i-Butyl		Numerous	Extinct	....
P. aurelia Clone 4	Control	Pale, odorless. Few paramecia, somewhat starved.	Numerous	Very numerous	Few thin
	Methyl		Numerous	Very numerous	Few good
	Ethyl		Multiplying	Nearly extinct	Few thin
	n-Propyl		Extinct	....	....
	n-Butyl		Numerous	Nearly extinct	Numerous
	i-Propyl		Numerous	Numerous	Accident
	i-Butyl		Very few	Numerous	Numerous
P. caudatum Wild culture	Control	Colorless, putrid. The aggregates of paramecia have been well broken up. Extremely numerous.	Extinct	....	....
	Methyl		Extinct	....	....
	Ethyl		Few	Excellent	Numerous thin
	n-Propyl		Few	Very numerous	Numerous small
	n-Butyl		Very numerous	Few thin	Nearly extinct
	i-Propyl		Few	Very few	Few thin
	i-Butyl		Numerous	Excellent	Numerous thin

The Influence of Temperature on the Susceptibility of Paramecium to Ethyl Alcohol.—Within reasonable limits of constancy a given concentration of a given alcohol will narcotize a definite per cent. of the organisms in a particular culture in one hour. This fact makes it possible to compare quantitatively the narcotic action of various alcohols, and to study the modifying influence of physical conditions on the effects of a particular alcohol. The method devised for counting the paramecia "narcotized" and those "unaffected" is elsewhere described (Bills, 1923*b*).

In the present experiment counts were made at widely different temperatures—8° and 25°. *Paramecium caudatum,* Clone 10, and 3.0 per cent. ethyl alcohol were used, and all observations made in duplicate. From the data presented in Table IV. it appears that the per cent. of the paramecia narcotized at 8° does not differ significantly from the per cent. at 25°. It seems improbable (though of course possible) that intermediate temperatures would show any markedly different values.

Table IV.

Showing the Influence of Temperature on the Susceptibility of Paramecia to Ethyl Alcohol.

The experiments were conducted in darkness.

Temperature.	Number of Paramecia Narcotized.	Number of Paramecia Unaffected.	Per Cent. of Paramecia Narcotized.
8°	50	793	5.9
8°	43	473	8.3
25°	47	568	7.6
25°	32	358	8.2

Average per cent. narcotized at 8° = 7.1.
Average per cent. narcotized at 25° = 7.9.

The Influence of Light on the Susceptibility of Paramecium to Ethyl Alcohol.—Pairs of burettes containing paramecia of Clone 10, with and without 3.0 per cent. ethyl alcohol were kept for one hour in strong, but diffuse, northern, daylight, or in direct, brilliant, sunlight in the middle of April. The direct light passed obliquely through the thin glass walls of the burettes. In all experiments the temperature was between 21° and 23°.

The data are presented in Table V. This table shows that direct sunlight inactivated in one hour 28 per cent. of the organisms in plain spring water, but that under like conditions, except that 3.0 per cent. ethyl alcohol was present, the sunlight inactivated 42 per cent. The experiments were repeated in diffuse daylight, and not one individual was inactivated in the absence of alcohol, while with 3.0 per cent. alcohol 8.9 per cent. were affected—a figure not significantly different from the values got in the temperature experiments which were made in darkness.

Table V.

Showing the Influence of Light on the Susceptibility of Paramecia to Ethyl Alcohol.

Light.	Per Cent. Alcohol.	Number of Paramecia Narcotized.	Number of Paramecia Unaffected.	Per Cent. of Paramecia Narcotized.	Average.
Diffuse daylight....	0.0	0	1361	0.0	0.0
Diffuse daylight....	3.0	213	1641	12	
Diffuse daylight....	3.0	28	454	5.8	8.9
Direct sunlight.....	0.0	558	1432	28	
Direct sunlight.....	0.0	451	1150	28	28
Direct sunlight.....	3.0	888	914	49	
Direct sunlight.....	3.0	726	1306	35	42

The Combined Effect of Preliminary Aëration and Agitation of a Paramecium Culture on its Subsequent Susceptibility to an Alcohol.—Aëration was effected by agitating for two minutes some paramecia of Clone 10 in 1.6 per cent. of *i*-propyl alcohol. This alcohol was chosen because of the fine froth produced when cultures containing it are violently shaken. The results presented in Table VI. show that the aërated paramecia are decidedly less susceptible than normal controls. Of the agitated organisms 17 per cent. were narcotized, whereas 32 per cent. were narcotized in the non-aërated control culture.

Table VI.

Showing the Influence of Aëration and Agitation on the Susceptibility of Paramecia to 1.6 per cent. *i*-Propyl Alcohol.

Treatment of Culture.	Number of Paramecia Narcotized.	Number of Paramecia Unaffected.	Per Cent. of Paramecia Narcotized.
Agitated and aërated....	239	1139	17
Normal control.........	682	1460	32

The Question of the Adaptation of Parmecium to Alcohol.—How does a preliminary exposure of *Paramecium* to a low concentration of ethyl alcohol affect the subsequent resistance to a stronger dose of ethyl alcohol, and of its homologues?

Towle (1904), working with electrolytes and simple organic compounds, concluded that "paramecia become readily habituated to solutions in strengths which are not soon fatal." Daniel (1908) found that paramecia when transferred *gradually* into distilled water become adjusted to this otherwise deadly substance. Estabrook (1910) developed in *Paramecium* a temporarily increased tolerance for strong doses of sodium chloride. Neuschlosz (1921*a*) found that *Paramecium* can develop a high resistance to dyes of the thiazin, benzidin, and triphenylmethane series. Neuschlosz later (1921*b*) reported that paramecia acclimatized to trivalent arsenic are at the same time resistant to trivalent antimony. Woodruff (1908) observed that alcoholized paramecia become more sensitive to copper sulphate. Their behavior toward a stronger dose of *alcohol* was not recorded. A case of adaptation in *Spirostomum* and *Stentor* reported by Daniel (1909) is of special interest, inasmuch as the method of experimentation is essentially identical with my method on *Paramecium;* the results, however, being different from mine. Daniel claims that he sometimes produced in these protozoa a slight adaptation to ethyl alcohol, but that this was accompanied by an increased susceptibility to methyl alcohol.

My experiments were made as follows: To 10 cc. of the Clone 10 culture taken from near the surface 10 cc. of 2.0 per cent. ethyl alcohol was added, making a 1.0 per cent. solution of alcohol. This mixture was put into a 30 cc. Stender dish and kept at approximately 24° for three days. At the end of this period the paramecia were observed to be distributed thruout the medium, appearing healthy, and distinctly more active than the controls altho possibly somewhat thinner. They were then exposed for one hour to each of the six alcohols in the concentrations indicated in Table VII., using quantities large enough to eliminate practically all error resulting from the presence of the original ethyl alcohol (see Bills, 1923*a*).

The results obtained are presented in Table VII. In this table the values given for the untreated controls are interpolated averages obtained for the six alcohols in a series of experiments elsewhere described (Bills, 1923*b*). They were made a few days before the present experiment was performed, during a period of extended constancy in the cultures. Therefore, these values are admissible for comparison here, and are probably more nearly accurate than single observations would have been.

From Table VII. it is clear that the three-day exposure to 1.0 per cent. ethyl alcohol increased the susceptibility of the paramecia to a narcotizing concentration of ethyl alcohol; and, similarly, to each of the other five alcohols. In other words, paramecia are not acclimatized to ethyl alcohol under the conditions of this experiment. Unlike Daniel's spirostoma and stentors which under similar conditions became more resistant to ethyl alcohol, paramecia became more susceptible to *all* alcohols.

TABLE VII.

SHOWING THE EFFECT OF EXPOSURE TO ALCOHOL ON THE ACTION OF ALCOHOLS ON *Paramecium*.

Period of Acclimatization to 1.0 Per Cent. Ethyl Alcohol:	Narcotizing Alcohols.	Per Cent. of Treated Paramecia Narcotized.	Per Cent. of Untreated Paramecia Narcotized.
72 hours.........	Methyl, 5.0%	Mostly disintegrated	30
72 hours.........	Ethyl, 3.3%	Many disintegrated	24
77 hours.........	*n*-Propyl, 0.9%	35	31
77 hours.........	*n*-Butyl, 0.5%	44	31
79 hours.........	*i*-Propyl, 1.6%	55	29
79 hours.........	*i*-Butyl, 0.4%	54	35

I wish to express my appreciation of the interest and guidance given me in the course of these experiments by Professor S. O. Mast and Professor H. S. Jennings; and my thanks to many other persons for their assistance in many ways.

SUMMARY.

1. A mixture of *B. lactis aerogenes* and *B. aquatilis* (sp. ?) constitutes the best food found for *Paramecium*.

2. When paramecia are exposed to an alcohol in sufficient strength they are at first incoördinated in movement and then inactivated. Later toxic effects are manifested by marked in-

ternal changes, formation of "blisters" by elevation of cuticle, rupture of ectoplasm, and death.

3. The anterior end of *Paramecium* is more susceptible to alcohol than the posterior end, and the aboral side more than the oral.

4. Indirect daylight has no perceptible effect on normal or alcoholized paramecia, but direct sunlight inactivates them; this effect is augmented in the presence of alcohol.

5. Change in temperature over a wide range has no appreciable effect on the susceptibility of paramecia to alcohol.

6. Aëration and agitation of a *Paramecium* culture renders the paramecia much less susceptible to alcohol.

7. Paramecia in a given solution without food live longer with alcohol than without; starving cultures can even be restored to prosperity by the addition of suitable amounts of any alcohol.

8. Exposure of paramecia to weak ethyl alcohol increases their susceptibility to a stronger dose of ethyl alcohol, and to five other alcohols.

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BIOLOGICAL BULLETIN

REACTIONS OF THE LARVÆ OF THE SHRIMP, *PALÆMONETES VULGARIS*, AND THE SQUID, *LOLIGO PEALII*, TO MONOCHROMATIC LIGHT.

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Since the larvæ of the shrimp, *Palæmonetes vulgaris*, and the squid, *Loligo pealii*, react to light very positively, it seemed interesting to discover what portion of the spectrum is most effective in stimulating them. For this purpose experiments were performed at the laboratory of the U. S. Fish Commission during the summers of 1920 and 1921 and at the Marine Biological Laboratory, Woods Hole, Mass., in the summer of 1923. The writer is indebted to Dr. S. O. Mast for valuable suggestions and criticism.

METHOD.

It was found that when the larvæ of the shrimp, *Palæmonetes vulgaris*, and the squid, *Loligo pealii*, are exposed to light, they turn and move definitely in the direction of its source. When exposed in a square aquarium at the intersection of two beams of light of equal intensity and at right angles to each other, the larvæ tend to distribute themselves in approximately equal numbers on the two sides of the aquarium which are most highly illuminated. If, however, the light in one of the beams is of greater intensity than that in the other, more larvæ aggregate on the side of the aquarium toward the brighter light. In other words the larvæ act as a sort of living photometer. It seemed possible, therefore, to apply to these organisms the method described by Mast (1907, 1917) for testing the relative stimulating efficiency of light of various wave-lengths.

The larvæ were placed in a small square glass container (5 cm. x 5 cm. x 2.5 cm.); opposite one side was placed a 10-watt Mazda lamp in a light-tight box (5.5 cm. x 5.5 cm. x 9 cm.) with an opening on the side toward the aquarium. The opening was covered by a smoked glass which reduced the illumination to 4.4 candle power. This box was movable on a meter scale toward and away from the container. Opposite an adjoining side of the aquarium at right angles to this was another box (5 cm. x 5 cm. x 14 cm.) containing a 15-watt Mazda candelabra lamp, in front of which could be inserted monochromatic light filters; this box was in a fixed position with the light 10 centimeters from the aquarium. Both lamps were operated by a single switch so that the larvæ could be exposed simultaneously to light from the two sources. The lamp producing white light was moved back and forth on the meter scale until the point was found at which the larvæ distributed themselves equally with respect to the two sources of light. The white and the colored lights were then considered to be of equal stimulating effect. Black screens with openings somewhat larger than the source of light were so arranged as to minimize reflected light (Fig. 1).

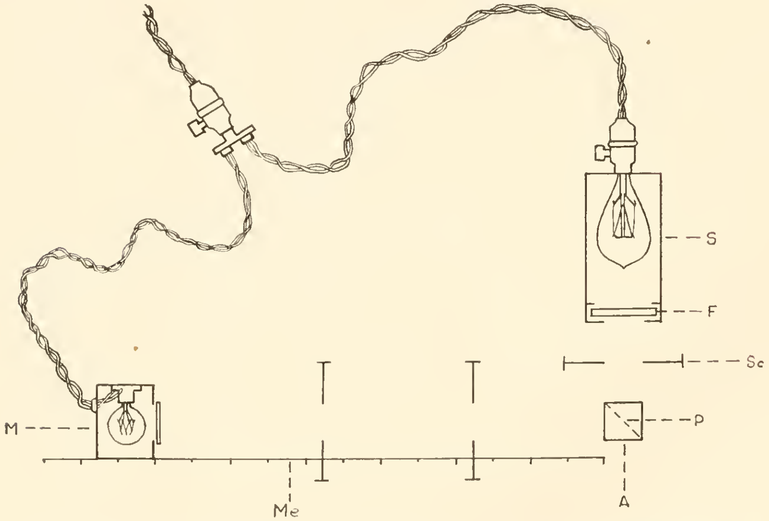

FIG. 1. Diagram of apparatus. *A*, aquarium; *F*, Wratten light filter; *M*, movable lamp; *Me*, meter stick; *P*, removable partition; *S*, stationary lamp; *Sc*, opaque screens.

Monochromatic light was secured by means of Wratten Light Filters Nos. 70, 71, 72, 74, 75, 76, and a combination filter composed of Nos. 22 and 53 made by Eastman Kodak Company. Each filter transmits a rather narrow band of the visible spectrum, most of the light being limited to a band 40 $\mu\mu$ wide or less. The per cent. of incident light of various wave-lengths transmitted by these filters has been measured by Eastman Kodak Company and is represented graphically by the white areas in Fig. 2.

The distribution of energy in the spectrum of the 15-watt Mazda lamp used in connection with the filters was ascertained by the U. S. Bureau of Standards (Fig. 3).

Since the distribution of the energy incident on the filters (Fig. 3) and the portion of this energy transmitted by each (Fig. 2) was known, and both had been ascertained for bands 10 $\mu\mu$ wide, it was possible to compute the relation in the energy of the light transmitted by the different filters in conjunction with the lamp in 10 $\mu\mu$ bands (Table I.).

Table I.

Relative Energy Transmitted by Different Wratten Light Filters with 15-watt Mazda Lamp.

Filter Number.	Relative Energy.
70 (red)	1176.0
71 (red)	225.0
72 (orange)	74.0
22 and 53 (yellow)	38.5
74 (green)	39.3
75 (green)	51.8
76 (blue)	10.35

In addition to the transmission in the visible spectrum all the filters transmit infra-red, but these rays were found to be ineffective for the larvæ of both *Palæmonetes vulgaris* and *Loligo pealii.* This was determined by subjecting them to light from which nearly all the visible rays were removed by means of Wratten Light Filter No. 88 which transmits only rays beyond 700 $\mu\mu$. Since the infra-red rays proved to be ineffective in stimulating the larvæ, they may be left out of consideration.

Ultra-violet was screened out by several layers of glass through which the light passed.

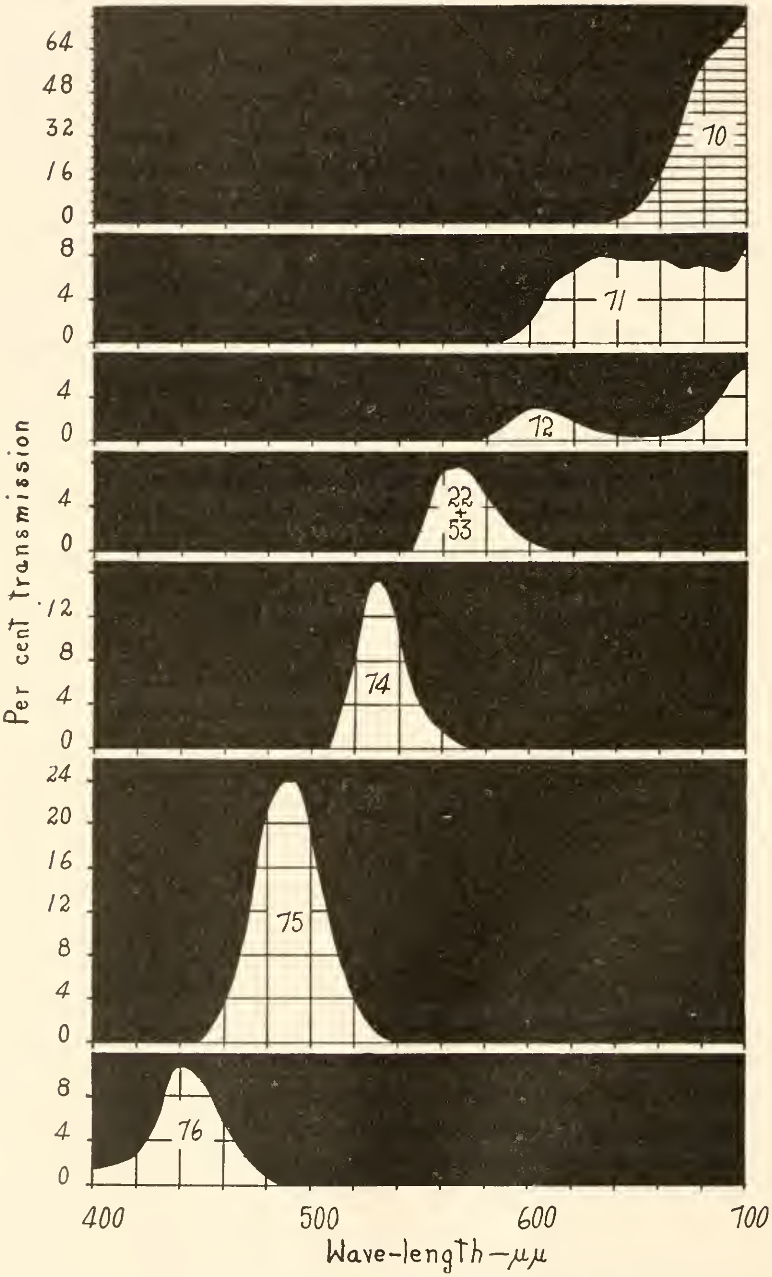

FIG. 2. Per cent. of light transmitted by Wratten filters used to secure monochromatic illumination.

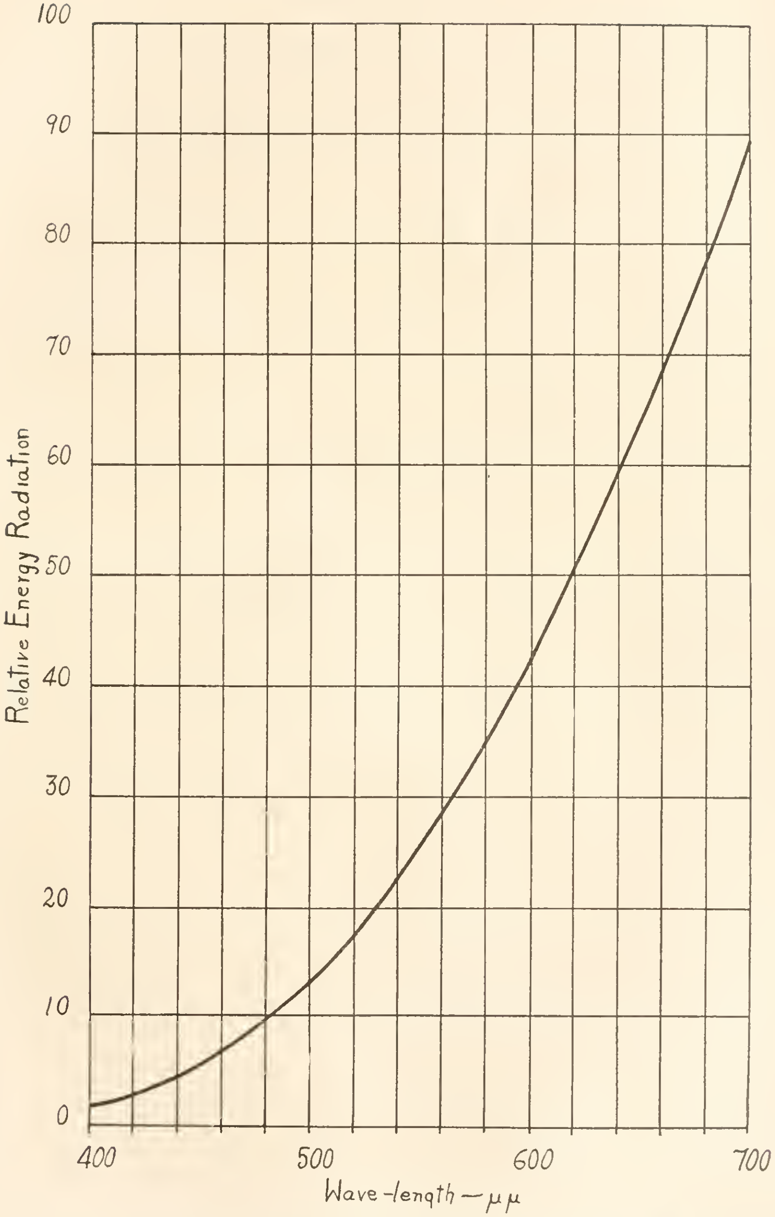

FIG. 3. Distribution of energy in the spectrum of the 15 watt Mazda lamp used, operated at 105 volts.

Experiments.

For both the shrimp and squid larvæ the experiment was performed as follows: A known number of larvæ, usually about thirty-five, were inserted into a small glass tube standing upright in the corner of the aquarium where the rays intersected, and allowed to remain one half minute in order to become dark-adapted. Both lights were then turned on simultaneously, and the glass tube carefully lifted out so as to free the larvæ without producing currents in the water. The larvæ were exposed for one minute, at the end of which interval a partition was slipped down into the aquarium in such a way as to separate the halves facing the two sources of light. The larvæ in each half were then counted and the number recorded; the light which had attracted the more larvæ was considered the more effective. In this manner for each filter numerous readings were taken with the movable lamp at various distances from the container, until the point was found at which the larvæ were equally distributed with respect to the two lights. At this point the stimulating effect of the light from the two lamps was, as previously stated, equal. The filters were found to differ in their effect in stimulating both the shrimp and squid larvæ, that is, for certain filters the movable lamp had to be placed nearer to the aquarium than for others, in order to obtain an equal distribution of the larvæ in the two halves of the container. Table II. and Fig. 4 summarize the results obtained.

Results and Conclusions.

In this table is shown the relative stimulation of the light transmitted by the different filters. Since the stimulating effect of the light transmitted by each filter is equal to the light produced by the movable lamp when this is so placed that the larvæ distribute themselves in equal numbers in the two halves of the aquarium facing the two sources of light, the relative stimulation of light transmitted by the different filters can be expressed in terms of the intensity of the light produced by the movable lamp. This is inversely proportional to the square of the distance of the movable lamp from the aquarium. These values are given in Table II. In order to translate these values on to the basis of relative energy, red filter no. 70 was taken as unity with a

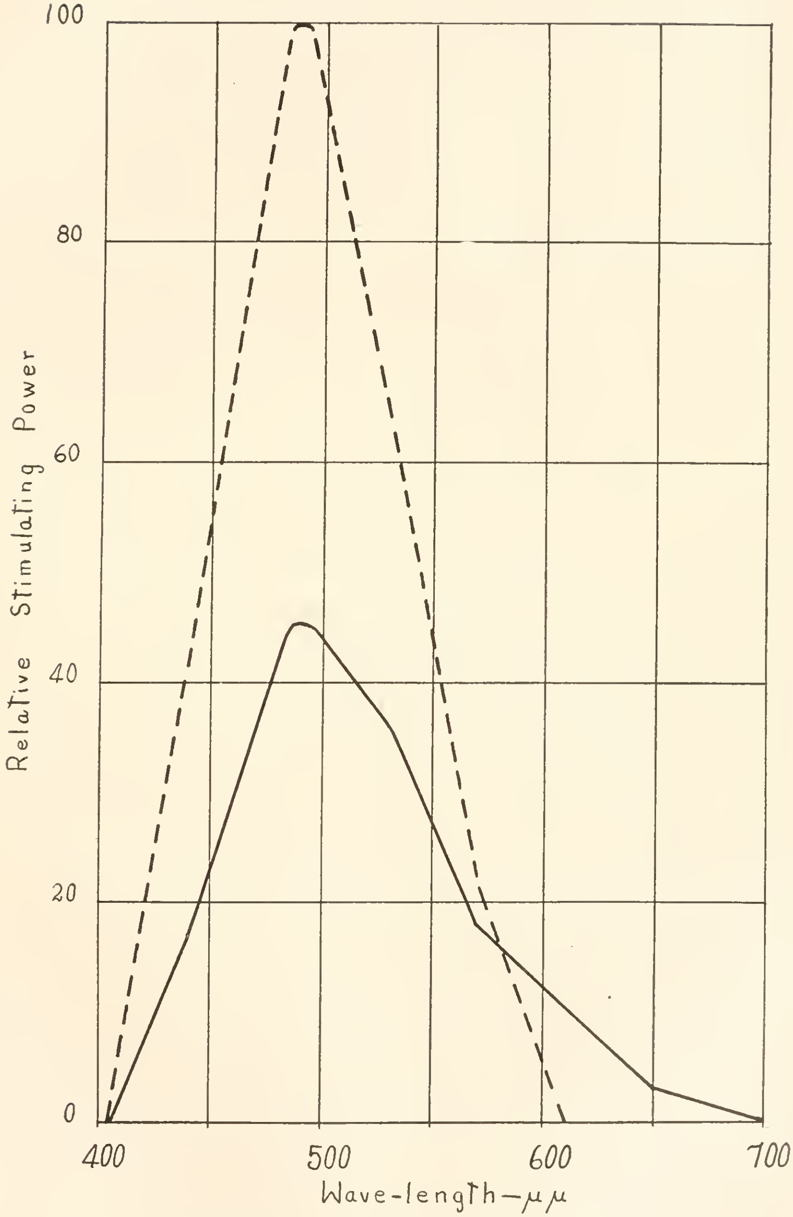

FIG. 4. Distribution of stimulating efficiency in the spectrum for shrimp and squid larvæ. Solid line, shrimp larvæ; broken line, squid larvæ.

relative energy value of 1176 (Table I.), and this number was divided by the relative energy value for each of the other filters. The resulting quotient in the case of each filter was then multiplied by the value for the relative stimulating effect of the light for that filter, thus giving values for the relative stimulating effect of light of equal energy content.

TABLE II.

RELATIVE STIMULATION OF LIGHT TRANSMITTED BY DIFFERENT FILTERS.

Wratten Filters.	Shrimp Larvæ.		Squid Larvæ.	
	Illumination.	Corrected For Energy.	Illumination.	Corrected For Energy.
Red No. 70	4.95	4.95	0	0
Red No. 71	2.77	14.46	0	0
Orange No. 72	1.38	24.88	0	0
Yellow Nos. 22 and 53	3.3	100.65	4.0	122.0
Green No. 74	6.25	201.25	11.11	356.63
Green No. 75	11.11	252.2	25.0	560.0
Blue No. 76	.83	94.29	2.04	231.74

The results obtained (Table II. and Fig. 4) show that for both the shrimp and squid larvæ the maximum stimulation is in the blue-green (470 μμ–510 μμ), and that yellow-green (520 μμ–550 μμ), yellow (550 μμ–590 μμ), and blue (420 μμ–470 μμ) are less stimulating. All these wavelengths are more stimulating for squid than for shrimp larvæ, particularly the blue-green and blue which are more than twice as effective. The rays beyond 620 μμ do not seem to effect squid larvæ at all, while shrimp larvæ, though not so powerfully stimulated, show some reaction.

Since both shrimp and squid larvæ possess eyes, it is reasonable to suppose that their reactions are due, to a large extent at least, to the nature of the photosensitive substances present in the eyes. Whether this is associated at all with the fact that the shrimp eye is compound while the squid has camera eyes would be interesting to learn.

Mast (1917) found from the study of fifteen species of various types that stimulation by light depended upon the wave-length. The distribution of stimulating efficiency in the spectrum differed in various forms without any apparent relation as to whether they were closely related species or not. It may be noted that in the majority of cases the wave-lengths which he found to be most stimulating were among those transmitted by the blue-green Wratten Light Filter No. 75, which was most stimulating for *Palæmonetes* and *Loligo*. The blue-green region was also found to be maximum in stimulating efficiency for a considerable number of different forms by Strasburger (1878), Engleman (1882), Verworn (1889), Wilson (1891), Gross (1913), Loeb and Wasteneys (1916), Laurens and Hooker (1920), Hecht (1921), and others. Various longer waves were, however, found to be most efficient in other species by Bert (1869), Lubbock (1991), Engelman (1882), Hess (1910), Loeb and Maxwell (1910), and others. It would appear, therefore, that the distribution of stimulating efficiency in the spectrum is not necessarily the same for all species having light-perceptive powers.

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FERTILIZATION, CORTEX, AND VOLUME.

OTTO GLASER.

I.

The initiation of development is associated with alterations in the cortex of the egg. Some of these changes are visible under the microscope; others are matters of inference. But writers on the mechanism of fertilization by no means agree in their interpretation of either the observed or the postulated phenomena. Indeed, there is uncertainty even in the realm of direct observation. For example, the fertilization membrane is considered by some as an epigenetic structure formed at the moment of impregnation, whereas others claim that fertilization merely results in changes by which a preformed membrane is rendered visible. Again there is the question of volumes. In 1913 and '14, I claimed for *Arbacia* as well as *Asterias*, an immediate loss of volume at fertilization; in 1921 Chambers claimed a constant volume and even an actual increase.

In themselves these details do not appear very important. It is not obvious just how current interpretations would be affected if it were possible to convince everyone either that the fertilization membrane is preëxistent or formed de novo. Yet with respect to volume, the case has practical consequences. It is true that here too, no compulsion, theoretical in origin, or otherwise, forces us to favor any particular assumption. Whether the egg increases, decreases, or remains constant does not inevitably involve any revision in theory. Of course, a decrease in volume as well as an increase might result from individual one-way mechanisms different in the two cases, whereas a constant volume might merely signify the absence of relevant changes of any sort. On the other hand, each of the three possibilities is conceivable as the resultant of antagonistic processes, one or more of which could be common to all. This, however, by no means implies that it is immaterial, practically, which of the three things is true. In our present state of knowledge, one of the three possibilities, it happens, is capable of making a more significant comment than the other two. For this reason, then, it is worthwhile to reexamine the question and to decide if possible whether the egg increases in volume, decreases, or remains constant.

II.

The question, unfortunately, has entanglements. Historically, as well as through needless misunderstandings, it is bound up with the problem of the fertilization membrane. This structure, despite the very clever criticisms recently made by Garrey ('19[1]) can be demonstrated on the surface of the unfertilized egg. After fertilization, the membrane demonstrates itself—a change brought about by easy stages in certain eggs.

In *Asterias*, for instance, the membrane, at ordinary temperatures, becomes visible rather slowly. In optical section, the perivitelline space is noticeable first as a small clear crescent at one point on the surface of the egg. From this region of initial visibility, the narrow area spreads in both directions until the horns of the crescent have met and completed approximately a circle. At this moment the egg is no longer in direct contact with the membrane whose diameter is now patently greater than before.

All this was implicit in my earlier statement ('13) in which I wrote: "the egg peels itself away from the inner surface of a thin preëxisting membrane. This peeling away seems to depend not upon changes in the fertilization membrane but upon changes in the surface film of the egg. When this is rendered more permeable, material leaves the egg and the egg shrinks away from its closely adherent covering which then becomes visible."

I applied the same interpretation also to the *Arbacia* egg though its perivitelline space is very much smaller. Quite recently the process of peeling has been very clearly described in *Echinarchnius* by Just ('19[2]). Chambers ('21) on the contrary, seems to have misunderstood my meaning. I did associate a shrinkage of the egg with fertilization and the appearance of the membrane; however I did not claim for either sea-urchin or starfish an exclusive monopoly of shrinkage or that "the initial diameter of the completed fertilization membrane is equal to that of the unfertilized egg" (*loc. cit.*, . . ., p. 332). Indeed, I can discover no relation between this statement and the facts, or my description of them. Material leaves the egg and the egg shrinks. These two processes appeared to me as intimately linked and for part of the time simultaneous. Furthermore I discussed the material which the egg eliminates ('14, p. 91). This secretion or excretion, as Loeb ('08) long ago rendered probable, contains a colloid to which the fertilization membrane itself is impermeable. The substance in question completes the conditions under which the perivitelline space can exert an osmotic pressure greater than that of the sea-water outside. Accordingly the membrane must become distended and its actual distension is so clear optically that no one before has ever doubted it.

Summarizing, we may say: the egg shrinks away from its enclosing membrane and the membrane becomes distended. These two movements in opposite directions account for the existence and volume of the perivitelline space.

III.

But does the egg actually shrink? Chambers denies the fact. The *Asterias* egg, he claims, gives no indication of volumetric decrease. If one waits long enough there is a measurable increase which I failed to notice because my "measurements were made on the assumption that the eggs always maintain a spherical shape." However the *Asterias* ovum is "very soft and if allowed to lie on the bottom of a glass dish tends to flatten into the shape of a disc," which "upon fertilization . . . rounds up as the fertilization membrane leaves its surface" (*loc. cit.*, . . ., p. 332). Under these conditions, observations taken in one plane would inevitably lead to erroneous conclusions—a deduction which is logically incontestible; which applies without question to the starfish egg at certain times and yet whose inapplicability to my work on *Arbacia* can be expressed quantitatively as 98.5 per cent.

For the sake of the discussion, I will assume that the eggs with which I worked were somewhat distorted by weight. It nevertheless remains true that in *Arbacia* gravity and surface forces quickly establish an equilibrium in which the unfertilized egg can maintain itself without appreciable change, for at least four hours ('14). During this time further movement toward or away from the spherical condition is negligible.

Under similar conditions the *Asterias* ovum changes markedly. The freshly shed egg is often pear-shaped and frequently flattened. It approaches the spheroid condition in the course of an hour in sea-water and it is then possible to select eggs not distinguishable from spheres. It was with such that I worked; they had established equilibrium and consequently were treated exactly like those of *Arbacia*. But in order to be consistent, I must assume that here again there was some flattening by weight and so for both sorts of eggs the question to be answered is the same: How much of the apparent decrease in diameter is the result of a change in shape and how much is indicative of an actual loss in volume?

The magnitude of the loss which I reported for Asterias is much greater than that found in *Arbacia*. However, all except twenty-five of my original measurements were based on the sea-urchin egg. In my search for further evidence, I therefore limited myself entirely to the second type.

The methods employed differed somewhat from the technique of Chambers. As before I limited myself to the period of actual fertilization. In these newer measurements I again preferred the stage micrometer to one of the ocular type. I also did not pierce the chorion with a needle or roll my eggs on gelatin plates in order to remove the jelly. In the absence of proof to the contrary, these procedures cannot be considered innocuous on account of the high salt content of the chorion ('22). In fact I used the chorion in order to avoid all possibility of discoidal distortion. This was accomplished by entangling the jelly on the minute fibrils of a slightly roughened cotton thread. In this way it is easily possible to prepare depression slides on which a considerable number of eggs have been ensnared by several fibers each one sticking to the jelly at a single point. This method has several advantages; the eggs remain "afloat" and move in the liquid whenever the fibrils bend; without being in contact with a solid surface, every egg if desired can be identified by its position along the thread; the attachment of the fibers at several points in the chorion corrects any distortion that might result from suspension at only one point. Furthermore the eggs in these preparations can be made to yield accurate records of volume and shape by projection in the form of silhouettes upon sensitized paper. The diameters of such prints can be measured by means of fine calipers and a vernier scale; while the exact magnification is made known by a similar projection of the scale of a stage micrometer. With the data thus secured the actual diameters of the eggs can be easily computed.[1]

In the following table (Table I.) no attempt has been made at the identification of individuals. The experiment is based on twenty-two eggs photographed before fertilization and again immediately after insemination.

Table I.

Diameters of Eggs.

Unfertilized.	Fertilized.
71.6 μ	74.8 μ
75.4	74.6
76.3	72.9
75.6	61.4
72.6	69.9
77.4	72.9
78.4	71.3
76.9	74.0
78.6	71.6
65.9	74.3
74.4	73.3
75.4	72.0
75.4	69.5
75.4	76.0
71.9	72.3
77.1	70.3
76.3	73.1
72.0	70.8
75.6	69.5
74.1	70.8
71.2	77.5
73.0	74.5
Average: 74.6	*Average:* 72.1

The result here—a loss of 2.5 μ is the same in sense as that found in a comparable mass experiment reported in 1914.

I next repeated the test on a smaller number of eggs whose identity as individuals, before and after fertilization was guaranteed by their position along the threads. The results are assembled in Table II.

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[1] For help in making these projections under the most favorable conditions, I am greatly indebted to Dr. Selig Hecht, who suggested the use of sensitized paper. Before I made the final measurements Dr. Hecht codified the prints and until I had his key to the system of labelling I could not by any means identify corresponding silhouettes. The measurements of the eggs therefore were made under circumstances which rendered the personal equation negligible.

TABLE II.

DIAMETERS.

Series.	Egg.	Unfertilized.	Fertilized.
A	1	74.1 μ	71.8 μ
	2	74.8	74.2
	3	73.3	71.4
	4	75.6	74.6
B	1	77.0	74.6
	2	75.8	73.7
	3	80.5	78.0
	4	78.2	72.9
	5	76.5	72.5
C	1	74.6	73.0
	2	72.9	72.2
	Average:	*75.7*	*73.5*

Here again the outcome is a loss of 2.2 μ.

This may be said to complete the crude verification of my earlier results. However there are suggestive discrepancies and this remains true whether we compare the mass experiments or those in which the eggs were kept identified throughout. Thus:

	1914.		1922.	
	Mass Experiment.	Individual Eggs.	Mass Experiment.	Individual Eggs.
Average Loss..........	3.4 μ	4.08 μ	2.5 μ	2.2 μ
Corrected.........		.. 3.5		
Total Average Loss		3.45 μ		2.35 μ

In this tabulation, I have averaged all the observations, but have introduced one correction in those of 1914. My original list of individual eggs, it happens, includes one in which the recorded loss was 11.8 μ—a figure which is probably wrong. Yet this elimination makes no essential difference. An absolute discrepancy in the magnitude of the losses reported now and in 1914 remains. This error, referred to the diameters of the original eggs, amounts to 1.5 per cent. Is this significant? Does it mean that the eggs of 1914 were to this extent flattened?

IV.

If so, it should be possible also to verify the results of Chambers provided only we can make the necessary observations under strictly comparable conditions. It will be recalled that Chambers "pierced the surrounding jelly with a needle." He did this in order that "the egg to be measured could be held suspended in the middle of the drop," hanging by one point from the ceiling of his moist chamber. Inasmuch as the starfish egg is "soft;" the jelly attached to the vitelline membrane; and this, prior to fertilization, to the plasma surface, the egg if distorted would, of course, approach a cylindrical form. Under the conditions described by Chambers, the observer would look lengthwise along the cylinder, and since the egg "rounds up" on fertilization, it follows that the initial measurement might be the diameter of a cylindroid and the final one that of a sphere. The second value should be greater than the first. But Chambers reports an immediate equality. One of two things then must have been true: either his eggs were not distorted—or the effect on the diameters produced by the change in shape was compensated by a loss in volume.

What is the likelihood that his eggs were distorted? It seems to me considerable for even the *Arbacia* egg under similar circumstances gives no indication of a decrease in diameter.

For these particular observations I selected eggs suspended from a single point. The measurements therefore were made under the conditions stipulated by Chambers. If now there is cylindroid distortion before fertilization, the "rounding up" process—which incidentally the *Arbacia* egg also exhibits—should give us a second measurement no smaller and possibly even larger than the first. The values recorded in Table III. speak for themselves.

TABLE III.

DIAMETERS OF EGGS.

Suspended at One Point.

Egg.	Unfertilized.	Fertilized.
1	76.3 μ	76.9 μ
2	72.3	72.5
3	75.7	76.0
4	73.5	74.2
5	72.9	74.2
6	74.4	74.2
Average:	74.2 μ	74.7 μ

V.

In accordance with these results then, the *Arbacia* egg by its own weight, is capable of flattening in sea-water. This vindicates the point made by Chambers. However, the extent of this flattening is negligible for the issues here at stake. This is shown by the measurements made under circumstances that leave no room for discoidal deformation. On the other hand, cylindroid distortion, when possible, may completely mask any losses in spherical volume.

We must now ask whether the loss here postulated anew for the *Arbacia* egg, is unique and whether it articulates well with the other facts of fertilization.

I did not repeat the work on the starfish because the difference there between the unfertilized and fertilized egg is even greater than in *Arbacia*. For *Nereis* too, special measurements, merely for the purpose of demonstrating a decrease in volume seem superfluous because the elimination of a voluminous mass of visible jelly is sufficient evidence. Again, according to Okkelberg ('14[2]) the egg of the brook lamprey is subject to a similar loss on fertilization.

These cases, of course, do not imply that losses everywhere must remain uncompensated long enough for us to measure them. In certain instances, alterations in shape may offset actual decreases in diameter; in others there may be compensatory swelling due to osmotic changes. Indeed, ultimately and no matter what may be true at the moment of fertilization, the egg increases in volume. The measurements which Chambers made 10, 20, and even 70, minutes after impregnation have no bearing on the immediate reorganizations of fertilization, however pertinent they may be in a study on growth.

But how do these losses in volume fit in with other facts falling within the fertilization period? In an earlier paper on egg-secretions ('14[3]) I compared the rates at which unfertilized *Arbacia* eggs and eggs in process of fertilization discolor the sea-water. The rates I found to be related as 2:3. During the summer of 1922 I repeated these observations in another way. On each of two small identical filters, I placed 1 cc. of a certain preparation of dry shed eggs. To the one filter was added a drop of sea-water; to the other a drop of dry sperm. The contents of the two filters were then gently mixed with a blunt glass rod, thus insuring a uniform distribution, in the one case of eggs and sperm; in the other of eggs and sea-water.

Although the experiment gave the expected result, it was not possible in this way to measure the loss of material from the eggs undergoing fertilization. For this, the filter papers and the capillary spaces between the papers and the funnels held back too much liquid. Nevertheless it was very evident that the amount of fluid secreted under these conditions by the inseminated eggs was markedly greater than that produced in the same time by the controls.

Evidently something leaves the eggs as they are undergoing fertilization—a fact which relates very easily to the observed decreases in diameter. In their turn these harmonize well with the observed cortical changes—themselves the basis of postulated and reasonably well attested increases in permeability. Indeed, we have here a system of closely interdependent events and for that reason I consider the loss in volume a matter of sufficient theoretical interest to warrant the expenditure of great care in those cases in which its demonstration is possible.

This opportunity the Arbacia egg seems to offer. The decrease in its diameter is not an illusion but the index of a loss in volume correlated with a real loss in mass; it also is not an oddity. Other eggs exhibit the same phenomenon. Furthermore the fact itself is not unintelligible. Indeed it may be related significantly and without violence to the entire constellation of events which together make up the initiation of development.

AMHERST COLLEGE,
November 25, 1923.

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VITAL STAINING OF AMŒBOCYTE TISSUE OF LIMULUS.[1]

LEO LOEB AND KENNETH C. BLANCHARD.

In our analysis of the factors underlying tissue formation,[2] it was found necessary to make use of vital staining in order to elucidate certain effects of environmental conditions on the cells and on amoeboid movement.

In the course of these investigations we made some observations on the staining of the amœbocyte; these observations may contribute to the understanding of the manner in which vital stains enter cells and are fixed or held back within the cells.

1. *Neutral Red.*—Two methods were used: (1) In the first one we prepared amœbocyte tissue in small stender dishes and replaced the supernatant serum by a solution of neutral red in a $n/2$ NaCl solution of the strength of 1:4000. After the tissue had been acted on by the staining solution for from one to several hours and had taken on a red color, it was used for tissue culture experiments. The specimens were kept for variable periods of time, usually in the icechest, but sometimes in the room, and examined daily during the next two or three or more days. The cells grew out of the piece in a centrifugal direction and gradually extended in the manner described in previous papers.[2] The pieces stained with neutral red either grew about as well as the unstained control pieces, or in some cases they seemed to grow slightly less well. In the amoebocytes, which had emigrated from the piece, the neutral red stain was usually localized in one or several droplets or particles in the interior of the cell. This condition applied to the cells which had spread out in a hyaline condition as well as to the more contracted granular cells. However, in some cases there were still some stained granules visible and occasionally we could observe such a granule, which had

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[1] From the Department of Comparative Pathology, Washington University, St. Louis, and from the Marine Biological Laboratory, Woods Hole.

[2] Leo Loeb, Washington University Studies, 1920, VIII., 3. *Science*, 1919, I., 502. *American Journ. Physiol.*, 1921, LVI., 140. *Sicence*, 1922, LVI., 237. Leo Loeb and K. C. Blanchard, *Amer. Journ. Physiol.*, 1922, LX., 277.

assumed the neutral red stain, entering a pseudopod. In a similar way in cells which had grown out into a $n/3$ solution of KCl, and in which circus movements developed in the amœbocytes, one or more stained granules participated in this circus movement in some cases. Even the droplets stained with neutral red seemed occasionally to enter a pseudopod.

Not only the cells which had grown out into Limulus serum and into neutral solutions of sodium chloride contained these droplets or particles of neutral red, but even cells which had grown out into acid ($n/1000$ HCl) and alkaline ($n/200$ NaOH) solutions of sodium chloride showed the typical neutral red droplets.

We have reason for assuming that originally the neutral red entering the cells stained the cell granules and that it was only secondarily deposited in droplets in the amœbocytes. The granule stain evidently had to a great extent disappeared at the time when the slides were examined. However, subsequently even the droplets, especially in the periphery of the field of outgrowth, disappeared in a number of cells in the course of several days. It seemed as if the droplets dispersed gradually into very fine particles and thus were ultimately destroyed. While, as we stated, the cells, as far as their staining with neutral red is concerned, did not at the time of examination show any difference in acid, alkaline and neutral solution, it is possible that later the droplets of neutral red disappeared somewhat more rapidly in the acid than in the alkaline solution; this, however, needs further examination.

However, in many cases the droplets remained unchanged even after the cells had been destroyed and the distribution of these neutral red droplets or particles indicated where cells had been previously.

We made use of this method of staining pieces of amœbocyte tissue with neutral red, in order to study the behavior of cells coming simultaneously from two different pieces of amœbocyte tissue and moving towards each other. For this purpose we placed two pieces, one stained and the other unstained, side by side on a cover glass. Where the two pieces came nearest to each other, the zone of outgrowing cells from both joined and formed a bridge connecting both pieces.

On the whole the tissues derived from the red and from the unstained piece, each respected the area of the other, but a number of red cells grew into the unstained area in a direction contrary to the centrifugal direction of the majority of unstained cells, and some of the latter grew similarly into the red area. Sometimes the cells moved until they reached the other piece and finding here resistance they turned around and moved back in the direction of their own piece. The cells were able to extend in the strange area and generally underwent the same changes as in their own territory. On the whole, the cells wandering out from the unstained piece remained unstained and did not take up neutral red, which may have become dissolved in the solution surrounding the pieces; but in a few instances, it is probable that such a secondary staining may have occurred in a few cells.

2. In the second method, we allowed tissue to grow out into the surrounding fluid, according to the cover glass tissue culture method, and after a sufficient layer of tissue had thus had a chance to form, we poured off the fluid surrounding the cells and replaced it by an isotonic solution of neutral red in sodium chloride. After this staining solution had acted upon the cells for from one to several minutes, we replaced it by a new solution which was free from stain. We have discussed the results thus obtained in another connection[3] and we shall here merely summarize some of our observations.

Almost instantaneously the neutral red penetrates into the cells and stains the granules red brown. In case the tissue had previously grown out in Limulus serum, the granules of the amœbocytes stain more deeply than if the tissue had grown out in a solution of sodium chloride.

Gradually the granules begin to lose their stain; instead of adhering to the granules, the stain begins to collect in the interior of the cells in the form of droplets or particles which are identical with those described above. If we add a weak acid (*n*/1000 HCl) to the outgrown and previously stained tissue, the granules lose their stain almost immediately. Furthermore, very soon the cells contract in this medium and cease to show amœboid activity. If we replace the acid by a weak alkali, the granule stain usually returns at least in the peripheral cells and a typical sequence of amœboid activity occurs. A second change of acid and alkali has the same effect as the first change.

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[3] *Am. Journ. Physiol.*, 1924, Vol. 67, 526.

This method permits us to observe more completely the effect of the stain on the cells, while in the first method we observed only the later changes.

We see then that acid and alkali have a very marked effect on the decolorization of the granules previously stained with neutral red and these observations suggested to us the experiments reported below on the decolorization of tissue as a whole in acid, alkaline and neutral media; this latter method makes possible a demonstration of the effect of hydrogen ion concentration on the stained tissue without the aid of the microscope.

II. In addition to neutral red we tested the effect of some other stains on amœboid tissue; for this purpose we made use of the first method. We stained the amœboid tissue in toto and used pieces of the stained tissue in tissue culture experiments. As usual the stains were dissolved in a $n/2$ solution of NaCl. On the whole our results with the stains other than neutral red were not very satisfactory.

(*a*) Methylenblue (1:4000), 2 to 2½ hours over tissue in stender dish. Hyaline, as well as granular, cells seem to take on a very slight, diffuse bluish stain; some granules show a more decided bluish coloration. Other cells are hardly stained at all. In some hyaline cells there are some blue droplets, which occasionally show a deeper coloration. There is still amœboid movement noticeable in these cells.

(*b*) Methylviolet (1:1000). In serum less outgrowth than in control; in $n/2$ NaCl very little outgrowth. Granules stain probably very faintly blue.

(*c*) Acriflavine (1:4000). The piece as a whole stains yellow, and the granules also seem to stain yellow.

(*d*) Eosin (1:500). The cells are unstained, although the tissue as a whole has a pink color. The cells grow out into serum as well as into a $n/2$ NaCl solution, but much less than in the control of unstained tissue. The injurious effect of eosin seems to be more marked in the sodium chloride solution than in serum; but even in serum some injury is noticeable. The cells grow out also into acid and alkaline solutions, but show apparently pathological changes.

(*e*) Trypanblue (1:5000). The cells appeared unstained. The outgrowth was similar to control.

(*f*) Janus green (1:4000). Appeared to be relatively toxic. There was apparently a very slight diffuse green stain; neither were granules distinctly stained nor were stained drops or particles visible in the cells. Some amœboid movement was noticeable in this tissue.

III. The Effect of Acid and Alkali on the Decolorization of Amœbocyte Tissue.

Method.—Amœbocyte tissue was prepared in stender dishes in the way described previously.[2] The stain (dissolved in a $n/2$ NaCl solution) was poured over the tissue after the supernatant serum had been poured off. Unless otherwise stated, the stains were used in a dilution of 1:5000.

During the process of staining the tissue was kept in the ice chest. The stained tissue was washed with $n/2$ NaCl solution. Pieces of tissue were then cut out and placed in the small test tubes which contained the solution, whose extractive power it was desired to test. Before comparing the amount of stain given off by the tissue in the various solutions, the pieces of tissue were removed from the tubes and the solutions in the different test tubes brought to the same hydrogen ion concentration and the same volume.

Neutral Red Tissue.—Solutions of $n/200$, $n/500$, $n/1000$ HCl very readily extract the neutral red from the tissue; the extraction usually becomes noticeable to the naked eye within a period of from ten to fifteen minutes or even somewhat earlier. In solutions of $n/200$, $n/1000$ NaOH, not more than a trace of stain is given off. Even after remaining in the alkaline solution for 48 hours in the ice chest very little stain was given off by the tissue. In neutral solutions of $n/2$ NaCl likewise no or very little stain is given off. Neutral red tissue which has been immersed in $n/1000$ and in $n/500$ HCl for 48 hours, and has given off red stain to the surrounding fluid, yields as much color again as it did the first time, if transferred to a fresh acid solution. Other acids, like $n/1000$ benzoic, butyric, lactic acid, extract the stain as well as $n/1000$ HCl.

Eosin Tissue.—Within a few minutes eosin tissue gives off stain readily to the alkaline (*n*/1000 NaOH) solution, a small quantity of stain to the neutral (*n*/2 NaCl) solution and none to the acid (*n*/1000 HCl) solution. This is in accordance with the acid character of the staining radicle in eosin. Eosin differs from neutral red not only in the reversal of the action of acid and alkali, but also in the greater ease with which a neutral sodium chloride solution causes the movement of the stain from the tissue to the solution.

Amœbocyte tissue stained with other stains gives less definite results. Methylviolet is apparently extracted equally by all solutions. Trypanblue does not give off enough stain to make comparisons possible. Acriflavine (1:4000) stains amœbocyte tissue deep yellow; alkaline, acid and neutral solutions seem to extract the stain equally well. Nileblue, on the other hand, bahaves somewhat similarly to neutral red. Acid (*n*/1000 HCl in *n*/2 NaCl) extracts the greatest amount of this stain, *n*/2 NaCl extracts a small amount, but alkali (*n*/1000 NaOH in *n*/2 NaCl) extracts none.

We see then that there is a definite relation between the acid or alkaline character of the dye used, and the character of the solution, which is most effective in extracting the stain from the stained tissue. It is not possible to modify this result if, previous to staining the tissue, we treat it on the following manner: we first allow acid (*n*/1000 HCl) or alkali (*n*/1000 NaOH) in isotonic *n*/2 NaCl solution to act on amœbocyte tissue for a period of three hours. The tissue is then washed with *n*/2 NaCl until the washings are neutral to brom thymol blue, when it is stained with neutral red (1:2000) for two hours, and then again washed until the wash fluid becomes colorless. After such preliminary treatment we found that the tissue previously exposed to alkali stained much lighter with neutral red than tissue exposed to acid or control tissue. Against acid, alkaline and neutral solutions the acid and alkali tissue behaved similarly.

If such acid or alkaline tissue is stained with eosin instead of with neutral red, the tissue behaves exactly like ordinary eosin tissue: the eosin is extracted by alkali, slightly by a neutral solution and not at all by acid.

Effect of Heating Amœbocyte Tissue on the Extraction of Stain.

In various experiments amœbocyte tissue was heated to a temperature varying in different experiments between 60° and 75° for fifteen minutes. This temperature is sufficient to kill the cells; at the same time the heated tissue becomes soft. If such a heated tissue is stained with neutral red and pieces of the stained tissue are extracted with acid, alkali and neutral solutions of sodium chloride in the usual manner, stain is apparently given off in all three solutions. However, if we centrifuge these solutions, we find that in reality only the acid solution was capable of extracting stain from the tissue. In the neutral and alkaline solutions the stain had not actually been extracted, but the soft state of the heated tissue had rendered possible, in the three solutions, the distribution of fine particles of tissue, which were stained and this suspension of stained particles of tissue simulated a real extraction. Amœbocyte tissue killed through heating behaves therefore towards the extraction of neutral red stain like living tissue.

The same results are obtained if we stain heated tissue which had been exposed to acid or alkaline solutions previous to the heating; again, only the acid extracts the stain.

If previously heated amœbocyte tissue is stained with eosin, instead of with neutral red, it is necessary to repeat the process of washing the stained tissue in $n/2$ NaCl about 40–50 times in order to remove a surplus of stain which adheres to the tissue. Tissue thus prepared gives off the stain most readily in an alkaline medium, somewhat less in a neutral, and no stain is given off in an acid medium. The tissue behaves therefore in this respect like living unheated tissue. An exposure of the tissue to acid or alkaline solutions previous to the staining does not alter this result.

Extraction of Stain from Stained Eggs.

Unfertilized eggs of *Asterias* were stained with neutral red, centrifuged and thoroughly washed with a solution of $n/2$ NaCl. Samples, each of two cc., of such an egg suspension were treated with an isotonic acid ($n/1000$ HCl) or isotonic alkaline ($n/1000$ NaOH) solution. The suspension was shaken, centrifuged, the supernatant fluid pipetted into test tubes, and the volume and hydrogen ion concentration were made the same in all the tubes (corresponding to a $n/1000$ HCl solution). While the tubes, which contained the originally alkaline and neutral solutions, were faintly stained, the acid solution showed the deepest stain. In this case the destruction of a certain number of eggs caused by the procedure used may possibly have complicated the result.

Eggs heated to 50° or 100° for ten minutes and stained with neutral red, gave off the stain even in a neutral solution of sodium chloride. The effect of acid and alkali on the extraction could therefore not be determined in this case.

Corresponding experiments with eggs stained with eosin could not be carried out because unheated starfish eggs do not stain with eosin, and while eggs, heated to 75°, take on a pink stain with eosin, the quantity of stain taken up by the eggs is not sufficiently great to make possible comparisons of the extractive power of acid and alkaline solutions.

Extraction of Stain from Stained Filter Paper.

If filter paper is stained with neutral red, it behaves towards extraction like amœbocyte tissue. For the purpose of extraction the same solutions were used as in the case of amœbocyte tissue. The stain is readily given off in an acid, but not in an alkaline or neutral solution.

Filter paper stained with eosin gives off the stain readily to an alkaline solution, but only a very small amount is extracted by a neutral and none by an acid solution. In other experiments filter paper was stained with trypanblue and subsequently washed in running water for one hour, then shaken with isotonic solutions of $n/1000$, $n/500$ and $n/250$ HCl and NaOH as well as with a solution of $n/2$ NaCl. Small particles of filter paper were suspended in these various fluids. In accordance with the acid character of trypanblue strong alkali extracted the stain, but acid, neutral or weaker alkaline solutions did not.

DISCUSSION

1. The granules of amœbocytes stain readily with neutral red. However, this is only a temporary effect; very soon the granules begin to give off the stain and this loss is almost complete within the course of one or two days. The time at which this change occurs varies somewhat, in some cells it takes place much earlier

than in others. It also depends upon the solution by which these cells are surrounded. In neutral solutions of sodium chloride the large majority of the cells have lost their granule stain within the first 24 hours. But in addition to the granule stain there is a second state in which the stain is found in the cell. It appears in the form of droplets or particles which are usually situated more centrally than the majority of granules, many of which are located in the peripheral part of the cell. Often more than one droplet or particle is present and the size of these droplets varies in different cells. In these droplets or particles the stain is retained much longer than in the granules. They resist also the decolorizing effect of acid more successfully than do the granules. They may persist for some time even after the cells have disintegrated and in such cases they indicate the place where cells have perished. The stain must therefore be fixed much more firmly in these droplets than to the granules. The variations which we find in the number and size of these droplets and particles make it probable that these drops represent cell vacuoles, rather than definite organs, although, if the latter should be the case, it would not alter our conclusions. It is probable that a certain surplus of stain, which cannot be held by the granules, is eliminated into cell vacuoles. In hyaline cells this is the only state in which the stain is found. These droplike formations in which the stain occurs in amœbocytes have some similarity to the droplike formations in which vital stains of an acid character are found in certain kinds of cells. However, such an acid stain, as trypanblue, does not seem to be taken up to any noticeable extent by amœbocytes.

2. In order to reach the granules the basic neutral red must pass through the outer cell boundary and through the hyaloplasm. In the case of neutral red the cell protoplasm (hyaloplasm) proper is not stained, but certain other basic dyes may perhaps cause a very light diffuse stain. A diffuse stain has been definitely observed in infusoria.

As to the reason why in most cases the protoplasm does not stain under those conditions, we may assume that the affinity of the dye for the granule substance is much greater than for the intergranular protoplasm. In addition it has been assumed that processes of reduction may make the dye invisible in the cell.

As to the relation between the diffuse protoplasmic staining and the granule staining, two views have been expressed. (1) Both the staining of granule and the diffuse staining of protoplasm depend upon the same process, namely, the solubility of the dye in lipoids of the protoplasm as well as of the cell granules (E. Nirenstein [4]); and (2) the staining of granules and protoplasm differ, inasmuch as the diffuse staining of the protoplasm depends upon the lipoid solubility of the stain, while the granule stain is due to a chemical combination between the basic radicle of the dye and an acid constituent of the granule, which latter is presumably not of a lipoid character (W. v. Moellendorff [5]). One argument on which the second view is based consists in the difference in the effects of alkali and acid on the staining of the protoplasm and of the granules. Addition of weak alkali increases the solubility of the dye in lipoids and is therefore believed to favor a diffuse staining of the protoplasm by neutral red. Addition of acid, on the other hand, diminishes the solubility of the dye in lipoids and it is assumed by W. v. Moellendorff that this is the reason why acid prevents the diffuse staining without destroying the staining of the granules. Our experiments prove that the amœbocytes behave differently in this respect: addition of alkali intensifies, while addition of weak acid causes a rapid loss of the granule stain. The protoplasm is not noticeably affected, as far as its staining is concerned, by either alkali or acid. Our observations show therefore that the staining of the granules is affected by acid in the same way as is the protoplasm in certain other cases, and we would therefore conclude that a distinction between the staining of these two cell constituents cannot be based on the argument which we have just cited.

3. According to our observations weak acid decolorizes very rapidly the granules of amœbocytes previously stained by neutral red, while alkali intensifies the staining. As we have seen, the effect of acid and alkali on the staining properties of neutral red has been referred to the influence which acid and alkali exert on the lipoid solubility of neutral red.

On the other hand, Pelet and Andersen [6] have shown that the dyeing of wool by acid and basic dyes depends upon the hydrogen ion concentration in the staining solution. They explained this effect as due to the influence of the H and OH ions of the solution on the electrostatic charges of the wool, which latter determine the tendency of the substance to combine with dyes of the opposite charge. They assume this combination to be one of adsorption. Bethe [7] and Rohde [8] apply similar conceptions to the staining of living cells. According to these authors the reaction within the cell determines whether the cells stain with acid or basic stain, and the combination between dye and constituents of the cells has the character of an adsorption. These conceptions are in contrast to those of others who assume that the effect of salts, acids and alkalies on vital staining depends either on the effect of these substances on the permeability of the cell for dyes, or, on their effect on the character of the dye itself. While Bethe believes the combination between constituents of the cell and dye to be one of adsorption, Jacques Loeb [9] showed that proteins combine with acid and basic dyes in a way similar to their combination with ordinary acid and alkali; in both cases the combination is of a stœchiometric chemical nature. In accordance with the amphoteric character of proteid, the latter combines with a basic dye in an alkaline solution and with an acid dye in an acid solution. M. Irwin [11] finds that the entrance of the alkaline dye (cresylblue) into the cell sap of Nitella shows a quantitative relationship to the hydrogen ion concentration of the surrounding fluid, and that these relations can be expressed in an equation characteristic of a monomolecular reaction. She interprets these findings as indicating a chemical combination between the dye and a protein constituent in the cell sap.

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[4] E. Nirenstein, *Pflüger's Arch.*, 1920, Bd. 179, 233.

[5] W. v. Moellendorff, *Ergebn. d. Physiol.*, 1920, XVIII., 141.

[6] L. Pelet and N. Andersen, *Zeitschr. f. Kolloidchemie*, 1909, II., 225.

Our experiments show that the reaction of the fluid surrounding the stained tissue determines whether the stain remains fixed to the tissue, or, whether it leaves the tissue. We furthermore found that these effects are the same irrespective of the tissue constituent with which the stain had previously combined; it applies in the case of the combination of neutral red with the granules as well as in the case of the combination of eosin with other constituents of the tissue. Even tissue killed through previous heating behaves like living tissue as far as the giving off of the stain is concerned, and both behave similar to filter paper which has been stained with acid or basic dyes. On the other hand, a preceding treatment of the tissue with alkali or acid does not alter the effect of the reaction of the surrounding fluid on the decolorization of the tissue.

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[7] A. Bethe, *Biochem. Zeitschr.*, 1922, Bd. 127, 18.

[8] K. Rohde, *Pflüger's Archiv.*, 1917, Bd. 168, 411.

[9] Jacques Loeb, "Proteins and the Theory of Colloidal Behavior," New York, 1922.

[10] Marian Irwin, *Journ. Gen. Physiol.*, 1923, V., 727.

So far as these experiments show, it seems then that the staining of cell granules, and of other constituents of the cells in amœbocytes, is of a similar character to the staining of cellulose; the surrounding acid or alkali competes with the tissue constituent or cellulose for the dye. The most probable assumption which we can make is that the electrostatic forces of primary or secondary valencies determine the fixation of the dye to the tissue, and that acid and alkali compete with the tissue for the alkaline or acid constituent of the dye. In addition it is very probable that in certain cases acid and alkali influence the result by converting the proteins into salts in which the protein constituent becomes either kation or anion.

While this statement applies as far as the test tube experiments with stained tissue and the microscopic behavior of the cell granules are concerned, it does not apply to the microscopic behavior of the droplike structures in which neutral red is deposited in granular as well as in hyaline cells. These drops seem to a much greater extent to be independent of the reaction of the surrounding medium. We saw that the stain remains concentrated in these drops at a time when in neutral solutions the majority of the granules have already lost their stain. We saw furthermore that these drops remain intact for a relatively long period of time in alkaline as well as in acid solutions, although the latter bring about the almost instantaneous decoloration of the granules. The droplike shape of these structures indicates their liquid character; they represent therefore in all probability solutions of neutral red. It is, however, possible that in addition the dye is deposited also in solid form in certain cases.

These droplike structures may remain preserved at least for sometime, even after the cells have been destroyed, and thus they may indicate the former situation of cells. We must therefore assume that there must be some factor which prevents these drops from mixing with the surrounding fluid. Two possibilities exist in this respect: either they are surrounded by a protein membrane, or, they consist essentially of lipoid material in which the stain is dissolved.

However that may be, we may conclude that when dissolved in droplike structures the neutral red remains preserved at a time when the neutral red which stains the granules has become dissociated from the latter. We may furthermore conclude that stained granules do not, as has been assumed, represent solutions of neutral red, and that neutral red may therefore be present in the cells in at least two forms, namely, (1) in a chemical combination with the granules, or, (2) in solution in the droplike structures, and that thus the conditions in which the neutral red is deposited in these two cases differ one from another. It may be suggested that the second mode of deposition of neutral red, namely, that in droplet form, is similar to the storage of acid vital stains, like trypanblue and pyrrholblue, which occurs in certain tissues.[11]

Summary.

1. Neutral red, as a representative of the basic dyes, stains the granules of the amœbocytes. The stained granules lose their stain gradually in neutral and almost immediately in acid solutions. Alkaline solutions intensify the staining.

2. In addition to the granules, droplike structures in the amœbocytes are stained with neutral red. The latter are very much more resistant to decoloration than the granules. They do not give off spontaneously the stain as readily as do the granules, nor are they as readily decolorized under the influence of acid as are the latter.

3. The effect of acid and alkali on the neutral red stain in amœbocytes can be demonstrated not only in single cells microscopically, but also in the test tube if we use amœbocyte tissue previously stained with basic or acid stains. Acids cause the giving off of basic dyes, and alkali causes the loss of acid dyes which had previously combined with the tissue. This relation prevails notwithstanding the fact that acid and basic dyes stain

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[11] H. M. Evans and W. Schulemann, *D. med. Woch.*, 1914, XL., 1508.

different parts of the tissue. Neither heating nor a preliminary treatment of the tissue with acid and alkali alters this result.

4. We conclude from these experiments that vital stains may be taken up by cells in two forms, (1) through the electrostatic forces of primary or secondary valencies they may be attached to the granules or to the cell protoplasm, or, (2) they may be present in a solution in circumscribed areas of the cell. In the latter form they behave towards environmental factors in a way similar to solutions of the stain. It is possible that also the acid vital dyes exist in the latter form, whenever they are taken into a cell.

THE MANNER OF COPULATION IN A TURBELLARIAN WORM, *PLANARIA MACULATA*.[1]

ROBERT A. BUDINGTON.

During the summer of 1920, while observing rather large numbers of flatworms, *Planaria maculata*, the writer chanced upon a quite ideal opportunity for watching copulation habits in that species. The worms were in a watch-glass which was easily transferred to the stage of a binocular dissecting microscope, and the attitude and relation of the mating worms thus became readily noted. Later they were killed, while still copulating, with a hot corrosive-acetic mixture; and although the worms separated during this treatment, the fixation was rapid enough so that the penes of the killed worms were preserved in very protruded condition.

Referring to the incident in conversation with several zoölogists, it seemed that they had never observed turbellarian copulation, nor did they remember having seen it described. On looking for data regarding it, it has not been possible to find any account of it either in the larger treatises or in more extended special papers on flatworm anatomy and behavior. Curtis, '02, who worked upon the "Life History, Normal Fission, and Reproductive Organs" of this same species for three years, informs me that he did not observe copulation. His studies were so extended, both in numbers used and in period of time, that one is inclined to conclude that either the procedure is not very frequent or that it is of short duration in any given instance.

Since there seems this gap in the recorded descriptions of this worm's total behavior, the following paragraphs are offered as supplementing our knowledge of it, as well as indicating what may be the impregnation process in turbellarians in general; for if, indeed, the process has not been much or at all studied, there may exist even so elementary a question as to whether or not the short copulatory organ is protruded through the atrial pore at all;

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[1] From the Department of Zoölogy, Oberlin College, and the Marine Biological Laboratory, Woods Hole.

or whether the spermatozoa may not be deposited in the atrial cavity of each worm and an exchange of them be effected by simple apposition of the pores, as is essentially the case in Annelida, also hermaphrodite.

Contrary to the behavior of oligochæte annelids which adhere in pairs, with anterior ends pointing in opposite directions, two *Planaria maculata* mate with heads in the same direction (Fig. 1).

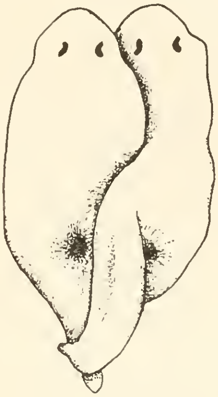

FIG. 1. Attitude assumed by two *Planaria maculata* during copulation.

The anterior ends are maintained side-by-side and flattened on the supporting substance (bottom of watch-glass in this case), oriented alike. About one third of their length posteriorly there begins a rather slight spiral twisting of the flat, oral surfaces of the worms against one another, so that the left ventral side of the right worm of a pair becomes lifted up against the right ventral side of the left worm. This twisting is carried further, posteriorly, so that the tail ends of the worms may even cross one another. At a point on the dorsal surface of each, opposite the external opening (genital pore) of the atrium, there was a marked depression caused by the extension of the penis directly underneath. This indicates that the penis of each worm is drawn into the atrial cavity of the copulating mate by a definite muscular grasp on the part of the walls of the enveloping atrium. This is also suggested by the narrowing of the proximal end of the extended penis; this is evident in Fig. 2. The relation of the two copulants is thus presumably as in Fig. 2; this is purely a diagram, however, and one may not infer that the penes necessarily lie laterally to one another always, for there may be no constancy in this detail of relations; the laterally side-by-side position, however, would seem better to permit approximation of each copulatory organ to the intra-atrial opening of the uterine duct of its mate.

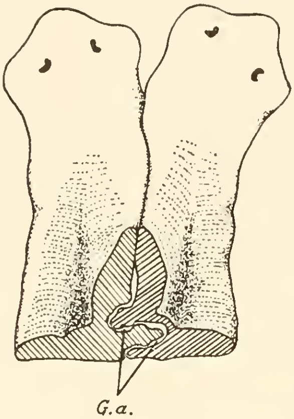

Fig. 2. Diagram of two mating worms cut transversely at level of the genital atria. *G.a.*, Genital atria.

Impregnation in this species is thus mutual and simultaneous between the members of a pair. The period of time over which they maintain this relation was not secured as it seemed desirable to fix the worms while copulation was in progress. Fig. 3 shows careful outlines of the two excopulants after fixation, showing relative size of body to copulatory organ, etc.

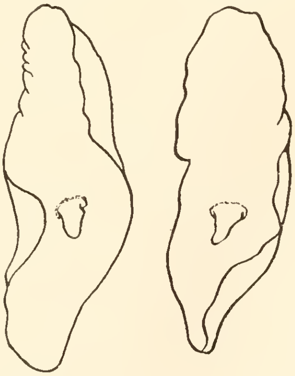

Fig. 3. Outline drawings of two ex-copulant flatworms after fixation. The penes still protrude.

To determine certain points relating to internal organs, serial sections were made of one of the pair; fixation proved to be satisfactory. As to the normal anatomy of the reproductive organs, the description and figures given by Curtis[1] are wholly adequate and correct. Naturally in the process of copulation the terminal portions of the vasa deferentia become adjusted to the protruded penis; and for the sake of easy comparison, Curtis's figure of the retracted arrangement of these organs is reproduced beside one drawn from the same aspect with the penis in copulating position. No comment seems necessary by way of interpreting these figures; in mating, the penis is turned posteriorly, extended through the atriopore, and considerably enlarged. This change in position tugs on the vasa deferentia (seminal vesicles) and straightens out the loops which occur in them when at rest.

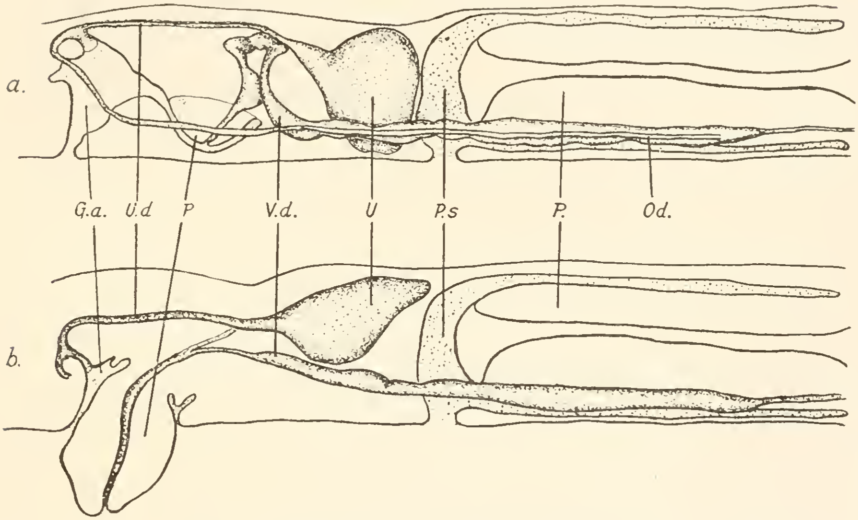

FIG. 4. (*a*) Normal resting position of the reproductive organs of *Planaria maculata*, from lateral aspect. (After Curtis.) *G.a.*, Genital atrium; *Od.*, Oviduct; *Ph.*, Pharynx; *P*, Penis; *U.*, Uterus; *U.d.*, Uterine duct; *V.d.*, Vas deferens. (*b*) Same as (*a*), with organs in position during copulation.

Concerning the place of deposition and storage of the transferred sperm, a further word may be added. The cavity of the atrium is considerably obliterated by the position of the penes; but the remaining space is more or less filled with spermatozoa, as is also the neck of the uterus, although this latter is very narrow and in the sectioned material it shows but a thin trail of sperm. As its wall is heavy with circular muscle, it is probable that it forced its contents along into the uterus while the fixation was still superficial. It also seems likely that few, if any, sperm are retained in the atrium after copulation is over; any such would be expelled through the external pore. The result of copulation will therefore be the reception of spermatozoa into the uterus of each copulant. This sperm mass in the uterus is (at least during copulation as here studied) in the form of a coiled cord or skein-like mass surrounded by mucus.

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[1] Curtis, W. C., *Proceedings of the Boston Soc. of Nat. Hist.*, Vol. 30, No. 7, 1902.

Gamble states in the section on Turbellaria in the Cambridge Nat. Hist., Vol. II., p. 38, that the copulatory organ when extended is long and narrow enough to reach up into the neck of the uterus; and that the eggs and sperm meet in that cavity. The present observations do not permit a denial of that statement; but the size of the organ as against that of the uterus duct as seen in Fig. 5 seems to make such an insertion, in *Planaria* at least, very improbable.

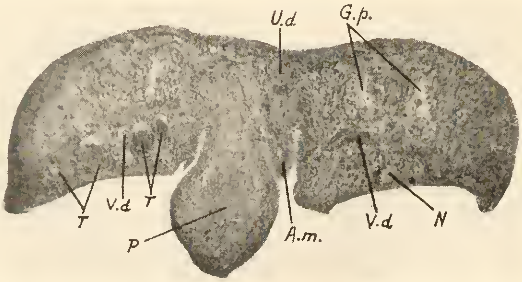

Fig. 5. Transverse section of *Planaria* at level of atrium, during copulation. *A.m.*, Atrial margin; *G.p.*, Gut pouches; *N.*, Nerve; *P.*, Penis; *T.*, Testes; *Ud.*, Uterine duct; *V.d.*, Vas deferens.

Again, although it is well known that in many forms the eggs are passed up into the uterus and meet the sperm there, in the present instance the uterus, as shown in sections, contains only sperm embedded in a mucus mass. The statement is often made that the sperm cells are aggregated into spermatophores; in a study of a single section this does seem to be the case, but further examination shows the spermatozoa to be in a long skein, much coiled, and surrounded by mucus. For a time at least the uterus may function merely as a sperm receptacle.

Summary.

1. *Planaria maculata*, and probably many other triclad Turbellaria, mate with both copulants oriented in the same manner.

2. Impregnation is mutual, and simultaneous.

3. During copulation the vasa deferentia are much distended by their sperm contents, while the uterus may act temporarily as a sperm receptacle only.

THE INFLUENCE OF HYDROGEN ION CONCENTRATION ON UNFERTILIZED *ARBACIA, ASTERIAS* AND *CHÆTOPTERUS* EGGS.

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In a previous paper the writers briefly reported experiments on the effects of increased H-ion concentration in sea water on the fertilization and development of the normally fertilized eggs of the star fish (*Asterias forbesii*) and sea urchin (*Arbacia punctulata*) (1). This work has since been repeated and extended in several directions and more complete reports on these subjects are now ready for publication. The experiments to be reported in this paper deal with effects of acid and alkaline sea water on the rate of ageing of unfertilized *Arbacia, Asterias* and *Chætopterus* eggs, and with the artificial activation of *Chætopterus* eggs by acid sea water. These experiments, beside affording a necessary basis for further studies of the influence of variations in H-ion concentration on the fertilization and developmental processes, furnish important information on the relation of the physiological activity of the egg cell to its environment.

Preparation of Acid and Alkaline Sea Water.

Certain of our experiments, which will be described in a subsequent paper, have shown that CO_2 exerts a profound effect, distinguishable from the effects of H ions, on many of the physiological processes of marine eggs. Since sea water naturally contains a considerable quantity of combined carbonic acid, it is necessary in all experiments designed to observe the effects of H ions *per se* to work with sea water from which the CO_2 has been removed. The CO_2-free sea water used in these experiments was prepared as follows: To each liter of fresh sea water was added 5 cc. of $N/2$ HCl and 5 cc. of $N/10$ NaH_2PO_4; this mixture was aërated with a water vacuum pump over night. No provision was made for excluding the CO_2 of the atmosphere because the concentration in equilibrium with the atmosphere after aëration is, so far as our experiments are concerned, negligibly small. *N*/40 NaOH was used for restoring this acid sea water to the desired H-ion concentration, using colorimetric standards with a salt content equivalent to that of sea water prepared as recommended by Clark (2) and McClendon (3). NaH_2PO_4 was added to the acidified sea water to give it greater buffering capacity in the neighborhood of neutrality, a rôle normally played by $NaHCO_3$. A sufficient quantity of acid sea water was made fresh for each day's use, and the individual solutions were prepared immediately before using.

CO_2-free sea water prepared in this manner was used between pH 4.5 and 8.0. In solutions more alkaline than pH 8.0 basic phosphates are thrown out, so solutions more alkaline than sea water were prepared by adding *N*/10 NaOH to natural sea water. In this case the carbonates do not interfere because the CO_2 tension is negligibly small. At pH 10.2 $Mg(OH)_2$ begins to precipitate; the amount of alkali required to complete this precipitation is many times the amount required to bring sea water from its normal reaction to 10.2. If the precipitation of $Mg(OH)_2$ were completed, the resulting solution would be physiologically unbalanced. It is, therefore, impossible to go beyond this point on the alkaline side. Consequently our observations are limited to the range pH 4.5 to 10.2, which embraces all the physiological variations with which we are concerned.

The addition of HCl and NaOH dilutes the salt content of the sea water slightly but we have no reason to believe that this slight degree of hypotonicity has introduced any serious complication into our results. Nearly all experiments reported in this paper were performed at approximately 20° C. Early in the season it was necessary to bring the sea water to this temperature, but later it was easily maintained with a slight regulation of the temperature of the laboratory.

THE VIABILITY OF *Arbacia* AND *Asterias* EGGS.

Asterias eggs were left in sea water until the maturation process was practically completed, during which time they were washed and gently agitated several times. *Arbacia* eggs were washed with sea water twice after removal from the ovaries and then used at once. The eggs were gathered into a concentrated suspension by gently centrifuging and a few drops of this suspension added to 200 cc. of the pH solutions contained in finger bowls. A small quantity of eggs was used so that there would be little crowding when the eggs settled to the bottoms of the bowls. During the exposure they were frequently agitated. At various intervals samples were transferred to sea water and inseminated with fresh sperm. The samples were examined after the eggs had had time to divide, noting both the number which fertilized, as shown by the presence of a fertilization membrane, and the number which had divided.

The results of several experiments with each species have shown that, as might be expected, there is some variation in the behavior of eggs from different individuals. The extremely artificial method by which the eggs are obtained—that is the excision of the ovaries and the consequent forced shedding—is not a method which could be expected to give eggs of uniform physiological quality from several individuals. But differences in the actual time of survival of eggs from different individuals, though important in some respects, do not materially alter the relative time of survival at different H-ion concentrations. Since the actual time of survival in any one experiment has no particular significance, it has been thought best to omit the extensive tabular data and to express the results in diagrammatic form with such quantitative expression as can reasonably be implied.

The relations between viability and H-ion concentration of *Arbacia* and *Asterias* eggs are given in Figs. 1 and 2. In these figures each contour line represents the proportion of eggs surviving at a definite interval after transfer to the pH solutions, the time of testing being marked on each curve. The data are based on the average results obtained from several experiments. The dotted curves marked "cytolyzed" show the proportion of cytolyzed eggs at the conclusion of the experiments. The full ordinate in these and all following diagrams indicates the H-ion concentration of sea water, *i.e.*, pH 8.15.

It might be supposed that marine eggs would retain for the

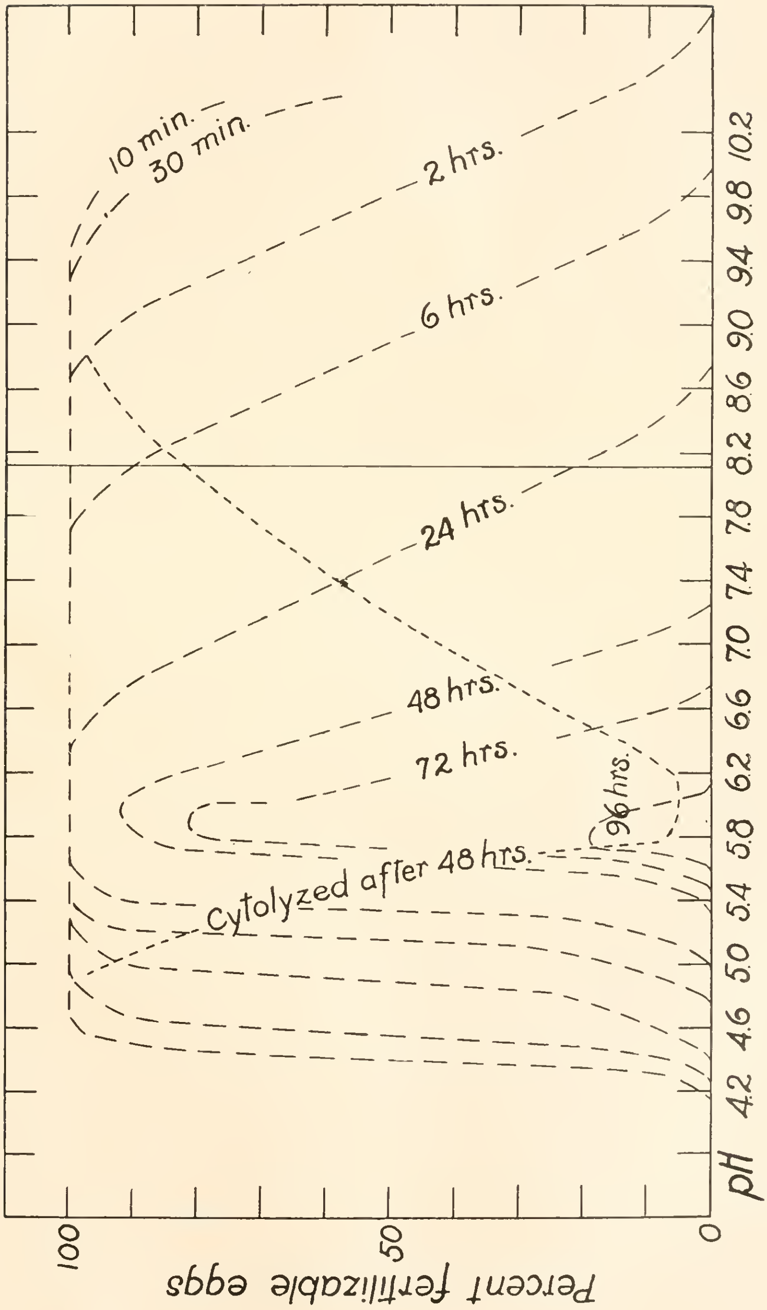

Fig. 1. The proportion of *Arbacia* eggs surviving after the stated exposures to acid and alkaline sea water.

longest time their capacity to fertilize (or to develop) in their normal habitat, sea water, the H-ion concentration of which rarely varies beyond pH 7.8 to 8.4. Contrarily, both *Arbacia* and *Asterias* eggs retain their viability longest, and are least susceptible to cytolysis, in quite acid solutions; the optimum reaction for *Asterias* lies between pH 6.2 and 6.6, and for *Arbacia,* 5.8 to 6.0. The eggs of both species die with great rapidity in very alkaline (pH 10.0) and very acid (pH 4.5) solutions; the rate of injury appears to decrease uniformly as the optimum is approached, particularly from the alkaline side. There is no disproportionate alteration in the rate of death around the pH of sea water. It will be noted that the optimum does not occur midway between the extremes but nearer the acid limits. That is, with respect to the optimum, the eggs of both species can tolerate a larger increase in alkalinity than in acidity. The *Arbacia* egg retains its fertilizing capacity much longer at all H-ion concentrations than does the *Asterias* egg.

It is well known that batches of *Asterias* eggs are frequently encountered in which all the eggs will not maturate or, even when maturated, will not fertilize. Ralph Lillie (4) has shown that the general physiological condition of such eggs could be greatly improved by treatment with ether, as shown by an increase in the number of eggs which both fertilize and divide. Similarly we find that after a short exposure to slightly alkaline sea water (pH 8.2 to 9.4), the proportion of fertilized eggs in these refractive lots is increased. This is expressed in Fig. 2 by the slightly higher incidence of fertilization on the alkaline side of sea water. This effect is clearly a more or less permanent alteration of the egg, since the eggs were transferred from the alkaline solution to normal sea water before insemination. It has sometimes been observed that the proportion of fertilizable eggs is decreased by a short exposure (3 to 5 minutes) to pH 10.0–10.2, and subsequently increased by longer exposures, short of permanent injury. No reason can be given for this apparent transient injury.

Goldfarb (5) has made careful studies of the consequences of ageing in sea water of three species of sea urchin eggs. *Arbacia, Hipponoë* and *Toxopneustes.* He finds that with increased ageing there is an increased tendency for agglutination, fusion of

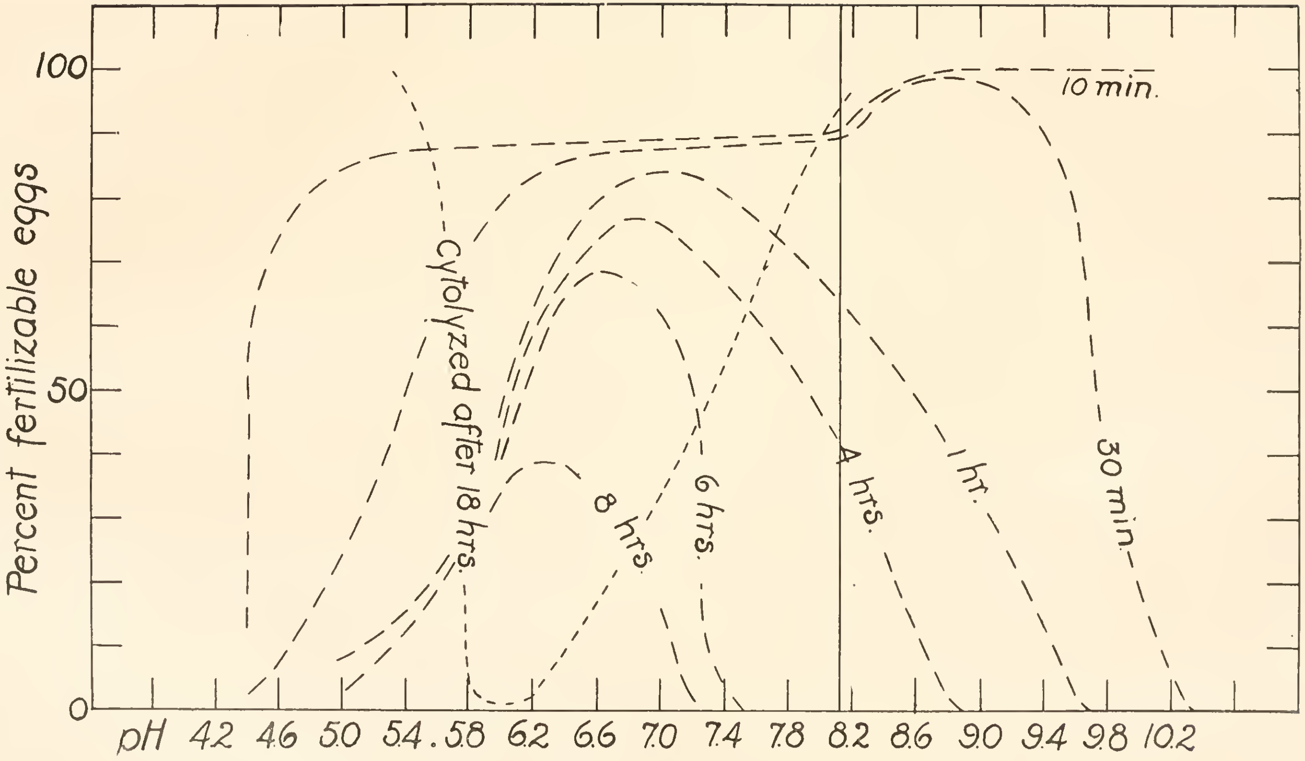

FIG. 2. The proportion of *Asterias* eggs surviving after the stated exposures to acid and alkaline sea water.

eggs and blastulæ, irregular cleavage as manifested in change of size and shape of the blastomeres, retardation in the rate of cleavage, and in extreme stages a total loss of the capacity to cleave. There is also an increase in volume and a loss of the jelly normally surrounding the fresh egg; in *Toxopneustes* and *Hipponoë* there is an initial acceleration in the rate of membrane formation, and in all three species a subsequent retardation of this rate and ultimately a complete loss of the capacity to lift a membrane. Goldfarb attributed the changes accompanying ageing principally to changes in the cortical layer, which, he states, are in turn referable to changed metabolism.

Our results confirm Goldfarb's findings in respect to the loss in *Arbacia* of the capacity to divide. This is equally true at all H-ion concentrations. In *Asterias,* however, on the alkaline side of the optimum the capacity to divide is lost before the capacity for membrane formation. But on the acid side of the optimum (pH 6.2 to 6.5) practically all eggs which lift membranes develop through the first few cleavages.

In both species the tendency for polyspermy increases in proportion to the physiological, rather than the temporal, age of the eggs. Consequently, the incidence of polyspermy is decreased at the optimum to about the same extent to which the viability of the eggs is increased. It is difficult to distinguish polyspermic from abnormally dividing eggs without cytological examination, and therefore it is deemed inadvisable to draw conclusions from our data concerning the tendency for polyspermy after ageing at various H-ion concentrations. It may be said, however, that among those eggs which are aged from pH 6.2 to 6.5 there is a decidedly lower incidence of both definite polyspermy and irregular division, as contrasted with eggs which are aged in more alkaline solutions. The former, if they divide at all, tend to divide regularly through at least two or three cleavages.

The ageing of *Arbacia* and *Asterias* eggs in sea water is accompanied by a slight increase in volume and fluidity. The nucleus which is difficultly discernible in the fresh mature egg, appears in the stale egg as a distinctly defined, hyaline vesicle near the center of the egg. Later, when the egg loses its fertilizing capacity, the cytoplasm becomes distinctly granular and opaque and the even contour of the egg is lost. As Chambers (6) has shown by microdissection, the granules in the dead egg are disintegrative products and not comparable to the granules in the living egg. They are no longer glutinous or adhesive; the egg has entirely lost its original homogeneity and is held together only by the investing vitelline membrane. Gradually this disintegrative mass imbibes water and swells within the vitelline membrane, becoming a more or less vacuolated liquid mass. Ultimately, the membrane breaks and the contents are dissipated in the sea water.

When *Asterias* eggs are allowed to age in acid and alkaline sea water, the transformation of the nucleus and the subsequent granulation of the cytoplasm occurs most rapidly in solutions more alkaline than sea water, and at about the same rate from pH 8.0 to 5.4. At acidities greater than pH 5.4 the nuclear transformation is perceptibly retarded and the cytoplasm acquires a granular appearance which differs from that of eggs aged in more alkaline solution principally by a diminished degree of discoloration.

From pH 5.4 to 6.2 many eggs are observed which contain, instead of a single vesicular nucleus, two, three or more smaller contiguous vesicles. Such eggs are observed much less frequently in solutions more alkaline than the optimum, pH 6.2. These polyvesiculated eggs will, when returned to sea water and inseminated, lift normal, turgid fertilization membranes in 3 to 5 minutes, and will usually cleave simultaneously into several blastomeres. If not inseminated when returned to sea water, a very small per cent. of the polyvesiculated eggs will fragment once or twice, the vesicles apparently being distributed among the fragments. Although the process of migration of these vesicles into the fragments prior to cleaving was not observed, they appear to be causally related to the process of fragmentation. Fertilization membranes are not formed spontaneously on the polyvesiculated eggs either in the acid by exposures of two to three hours, or when returned to sea water; though a few eggs will form fertilization membranes if left in the acid solutions for considerably longer periods, 4 to 6 hours. The fragments are held together by a delicate membrane bridging the furrows; this may be the vitelline membrane of the unfertilized egg.

The nucleus of the *Arbacia* egg acquires a vesicular appearance on ageing much the same as that of the *Asterias* egg. As in the latter case this change occurs in about the same time at all H-ion concentrations from pH 5.0 to 10.0. At acidities greater than pH 5.0 there is an increased incidence of eggs in which this nuclear change does not take place. We have not observed the appearance of several vesicles in the *Arbacia* egg at any H-ion concentration, but our observations would not preclude their existence.

In solutions more alkaline than pH 9.0 there is a tendency for membranes to lift spontaneously on both *Asterias* and *Arbacia* eggs. Prior to membrane formation the cortex of the egg undergoes peripheral disintegration with formation of droplets. Spontaneous membrane formation decreases with increasing acidity; below pH 7.0 it is rarely observed except when induced in *Asterias* eggs by long exposures to pH 5.4–6.2, as mentioned above.

CHANGES IN PHYSICAL PROPERTIES.

The appearance of eggs which have been exposed for a short time to extremely acid or alkaline sea water is markedly different. Alkali treated eggs present a smooth, almost glassy surface, while acid treated eggs are dull and appear to have a finely granulated surface. The slightest amount of manipulation indicates that the alkali treated eggs are soft and more liquid than normal, while eggs treated with acid are more solid. Dr. Chambers has kindly examined the effects of acid and alkali on these eggs by means of microdissection. He finds that in acid sea water the thin, delicate vitelline membrane which normally surrounds the unfertilized *Arbacia* and *Asterias* egg is toughened so that it is difficult to tear. This toughened membrane makes it difficult to ascertain mechanically what influence the acid solution may have on the consistency of the egg surface itself, but with non-injurious exposures acid seems to produce no very profound change in the consistency of the egg cortex. Longer exposures lead to a gradual setting or gelation of the cortex (and possibly the egg as a whole), finally accompanied by the loss of its normal transparency.

In alkali both *Asterias* and *Arbacia* eggs are rendered extremely plastic, soft and liquid by short treatment. This can be shown by shaking the eggs for a uniform time in solutions of increasing alkalinity. Thus when *Asterias* eggs are vigorously shaken in sea water of pH 5.0 to 10.2 after an exposure of 10 minutes, very little cytolysis or fragmentation occurs between pH 5.0 and 9.0. At pH 9.3 there is a slight amount of fragmentation and a slightly increased number of cytolyzed eggs. A large number of the eggs are distorted from their normally spherical shape, showing that they have softened. At pH 9.6 a few eggs are broken into smaller fragments and the number of intact but cytolyzed eggs is diminished as compared with 9.3. At pH 9.9 the membrane and egg cortex are abruptly destabilized and all the eggs are readily broken into small, spherical and extremely stable fragments.

Similarly the *Arbacia* egg is comparatively resistant to moderate shaking between pH 5.8 and 9.3. At about pH 9.6 there is a marked increase in the tendency to cytolyze. This egg does not fragment as does the *Asterias* egg, presumably because of its inability to form new surface films readily, but appears to cytolyze rather slowly after rupture at some one point. From pH 9.6 to 10.2 the shaken suspensions are filled with ghosts and partially cytolyzed eggs.

The Maturation of *Asterias* Eggs.

The maturation of *Asterias* eggs is normally initiated as soon as they are removed from the ovaries and come in contact with sea water. The initiating factor or factors are not known. Loeb (7) has shown, however, that the addition of acid to sea water inhibits, and the addition of alkali favors the maturation process. When slowed below a critical velocity the maturation process stops and the eggs remain permanently immature.

In view of the possible rôle of H- or OH-ions in initiating maturation, an examination was made of the effects of increasing acidity on the incidence of permanently immature eggs. The eggs were introduced into the pH solutions without contact with sea water by dipping small pieces of fresh ovary into the pH solutions. After 45 minutes or an hour counts were made of the mature and immature eggs, discriminating by the dissolution of the wall of the germinal vesicle. A summary of experiments of this nature is given in Table I.

Table I.

Nine Experiments on the Influence of H-Ion Concentration on Maturation of *Asterias* Eggs.

Per Cent. Permanently Immature.

pH									
6.0	6.2	6.4	6.6	6.8	6.9	7.0	7.2	7.4	8.15
	91	72	58	32	28	45	30	6	0
	88	20	44	40	23	40	12	4	0
99	90	82	43	26	8	27	5	1	0
	58	34	22	16	8	18	10	4	0
	76	80	68	63	48	50	35	31	0
	93	76	69	46	60	64	25	10	1
95	88	71	61	39	36	26	14	5	8
93	86	68	69	57	33	16	17	5	1
	76	20	28	33	39	21	17	4	5

Maturation is practically inhibited at pH 6.0, a point at which normally fertilized *Asterias* eggs will grow quite normally, and approximately the point at which the unfertilized egg retains its viability for the longest time. It should be noted in Table I. that the incidence of maturated eggs does not always increase uniformly with increasing alkalinity, but that in some experiments the proportion of immature eggs falls to a low value on the acid side of neutrality, rises noticeably at pH 7.0 (or 6.9) and then falls to zero at 8.15. This irregularity in the influence of H-ion concentration on the maturation process is not affected by washing several times in the respective pH solutions. It appears, therefore, to be attributable to alterations in the egg cortex rather than to the activity of some substance in the supernatant fluid.

The Influence of H–Ion Concentration on *Chætopterus* Eggs.

The *Chætopterus* egg differs from echinoderm eggs in that it is activated by sea water to which HCl has been added, as Loeb showed many years ago. This activation, though not qualitatively nor quantitatively sufficient to produce normal larvæ, makes it necessary to consider separately the consequences of exposing the unfertilized egg to acid solutions, and the effects of such exposures on subsequent fertilizability.

Like the egg of *Asterias,* the *Chætopterus* egg is shed immature; though the germinal vesicle breaks down when the egg is placed in sea water, maturation proceeds only as far as the formation of the first maturation spindle. Unless the egg is fertilized or artificially stimulated it remains in the metaphase of the first maturation division. After fertilization the maturation divisions are completed and the polar bodies are formed at the animal pole. Mead (8) first called attention to the fact that a small amount of KCl added to sea water causes these eggs to complete the maturation process with polar body extrusion, an event in this case signifying initiation of development. Three years later Loeb (9), having succeeded in obtaining parthenogenetic development of echinoderm eggs by the use of hypertonic sea water, tried similar procedures on *Chætopterus* eggs. He also examined the influence of KCl and concluded that activation in this case was not a consequence of increase in osmotic pressure, but a specific effect o' K-ions. In a careful description of the parthenogenetic development of this egg, he pointed out that after treatment with KCl, development and differentiation appear to proceed without cell cleavage (an observation subsequently confirmed by F. Lillie, 10), and that activation of the egg is accompanied by marked ameboid movements of the protoplasm. He did not succeed at this time in obtaining development with cleavage, though later in collaboration with Wasteneys he obtained cleavage by the combined use of $SrCl_2$ and ox serum. Loeb also decribed a marked tendency in *Chætopterus* eggs activated by KCl to adhere to each other; as a result of this adhesion, larvæ might be formed consisting of several swimmers partially fused, or in some instances, of giant swimmers resulting from the complete fusion of several eggs.

Loeb also obtained ciliated, unsegmented larvæ by treating *Chætopterus* eggs with 100 cc. sea water + 2 cc. $N/10$ KOH, and with 100 cc. sea water + 2 cc. $N/10$ HCl. He mentions the fact of activation by HCl as striking since he had failed to get activation of echinoderm eggs by similar treatment. (It was not until five years later that he tried the fatty acids with signal success.) Allyn (11) has recently examined the action of several acids on *Chætopterus* eggs. She failed to get segmentation and concluded that acids are less effective than KCl.

In our experiments with *Chætopterus* we have observed the effect of acid and alkaline sea water both in respect to activation and to the subsequent fertilizability after varying exposures. The worms were removed from their tubes as soon as they were brought into the laboratory, and the males and females were placed in separate dishes with running sea water. Before use the females were rinsed well with tap water, then with sea water, and placed in about 50 cc. of sea water in a finger bowl. The egg sacks were cut and the ovaries removed and gently teased apart. After about 15 minutes when all the ripe eggs were shed, the tissue fragments were picked out, the egg suspension filtered through cheese cloth and the eggs concentrated by centrifuging. Because of the small quantity of eggs available, it was necessary to reduce the volume of the pH solutions to 50 cc. Equal quantities of maturated eggs were added to each of the pH solutions; at various intervals portions of these eggs were transferred to two dishes of sea water, only one of which was inseminated. Fresh sperm from one male were obtained when desired by cutting a new sperm sack and allowing the escaping sperm to accumulate in a small quantity of sea water.

The activation of *Chætopterus* eggs by H-ions is illustrated in Fig. 3 by the dotted line. The activation is most intense at pH 5.8 and diminishes rapidly on either side of this point, practically disappearing at 5.0 and 6.6. If the eggs are left at pH 5.8 polar bodies are extruded in 30 to 50 minutes, and about 50 per cent. or more will at the end of two or three hours show marked ameboid movement and extensive fragmentation by budding. The egg flows spontaneously into several unsymmetrical pseudopodia and these in turn develop smaller extrusions, many of which bud off into small spherical fragments. Nuclear division apparently does not always precede this fragmentation, which seems to be largely a result of the ameboid activity of the cortex. If, however, the eggs are exposed for one or two hours to the acid solution and then returned to sea water, 50 per cent. or more will undergo one or two segmentations which more or less simulate the divisions of the normally fertilized egg. They do not develop beyond the two- or four-cell stage, however, and in the majority of instances the cleavages are irregular and the blastomeres tend to separate. If the eggs are returned to sea water after shorter exposures a still smaller proportion of them show signs of activation. A 30-minute exposure will produce polar body formation but only a few eggs will fragment. A 10-minute exposure is insufficient to activate at any H-ion concentration; attention is called to this point because this exposure produces changes in the egg which prevent fertilization by sperm.

There is also a slow activation in alkaline sea water, beginning about pH 9.6 and increasing to 10.2. The activation is apparently not so intense as at pH 5.8 since an exposure of 4 to 5 hours is required to induce segmentation.

It should be emphasized that the H-ion activation of *Chætopterus* eggs is not strictly comparable to the activation of echinoderm eggs by the fatty acids. Loeb showed that in *Arbacia* the strong acids were practically incapable of activating; the fatty acids are efficient by virtue of their penetrating power. Loeb obtained slight activation of *Asterias* eggs by treatment with sea water acidified with HCl (7), but we have obtained no activation of either *Asterias* or *Arbacia* eggs by CO_2-free sea water as acid as pH 4.5. It is probable that the activation obtained by Loeb was due to free CO_2, which Delage has shown to be an excellent activating agent (12).

If *Chætoperus* eggs which have been exposed to the pH solutions for 10 minutes are returned to sea water and inseminated, those from solutions in the neighborhood of pH 5.8 will not fertilize; they remain inert when the eggs taken from the solutions at pH 4.6 or 7.6 have fertilized and have undergone two or 3 normal cleavages. A 10-minute exposure to these solutions, though insufficient to activate, apparently produces some block to fertilization which is not produced by equal exposures to pH 4.6 or 7.6, or to the alkaline solutions which also activate. (The solid line in Fig. 3 shows the proportion of eggs which fertilized in sea water after an exposure of 10 minutes to the pH solutions.) But if the eggs are left in the pH solutions for 30 minutes or longer before being transferred to sea water and inseminated, they slowly recover their fertilizability, in as much as the addition of sperm causes them to segment normally to the 16- or 32-cell stage, and to develop into swimming larvæ of more or less normal appearance. The longer the exposure to the pH solutions, the greater the pro-

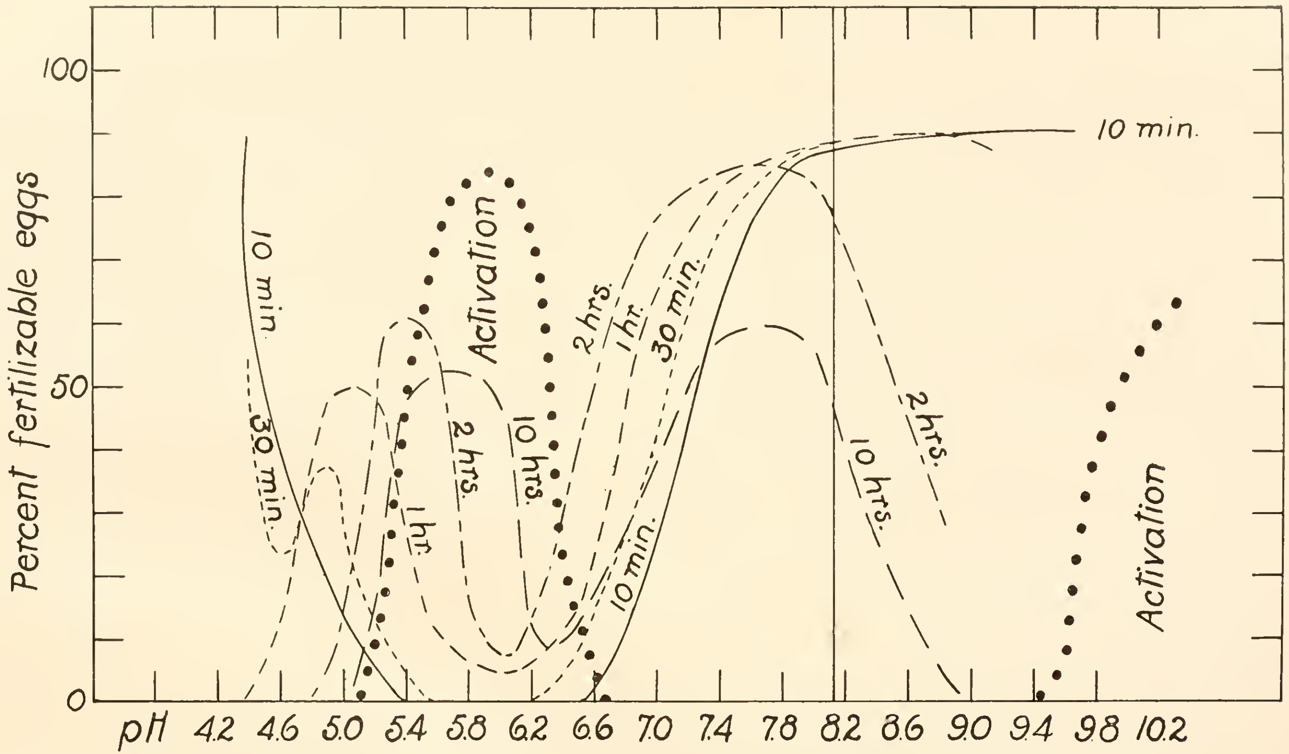

FIG. 3. The heavily dotted lines show the regions of artificial activation of *Chætopterus* eggs by acid and alkaline sea water. The solid and the dashed lines show the proportion of eggs which fertilize when returned to sea water and inseminated after the stated exposures to the pH solutions. A block to fertilization is created by short exposures to those solutions having the greatest activating power; when the eggs are left in these solutions this blockgradually disappears.

portion of eggs which recover their fertilizability. (The dashed contour lines in Fig. 3 show the general course of this recovery process. The figures written beside each curve show the duration of the exposure to the pH solutions.) The block to fertilization produced by short exposures to the solutions around pH 5.8 disappears only when the eggs are left in the acid solutions. If returned to sea water after a short exposure (*i.e.,* 10 to 30 minutes) the eggs remain permanently (4 to 6 hours) unfertilizable.

Dr. Chambers has examined these eggs by microdissection and finds that when treated for 5 minutes at pH 5.8 and then placed in sea water, the membrane which envelops both the fertilized and unfertilized egg is very much thickened and toughened. Longer exposure to pH 5.8 tends to soften this membrane so that when returned to sea water it is thin, delicate and easily torn. Though the conclusion is by no means substantiated, it is possible that this initial toughening of the membrane with subsequent softening on longer exposure accounts for the inability of sperm to react with short exposure as compared to long exposure eggs.

That the sperm gain access to the egg after the block has worn off is shown by the fact that without sperm they undergo at the most two or three cleavages which are decidedly late and irregular, while with sperm they develop with much more normal velocity and with a quality that is so nearly normal that in many instances they cannot be distinguished from normally fertilized eggs. Many of them, moreover, develop into rough swimmers. There is a marked tendency for the blastomeres to fuse in the later stages with the production of syncytia; and for the separate blastulæ to fuse, 4 or 5 forming one large, apparently homogeneous larva, or for several to adhere together forming irregular chains. This tendency for fusion, like the ameboid movements which accompany activation and normal division, is clearly a consequence of some lability of the cortex. Fusion appears to be more marked among those eggs exposed to solutions on the acid side of pH 5.8 than on the alkaline side.

If we neglect the temporarily irreversible block created at pH 5.8, the optimum reaction for the retention of viability will probably be somewhere in this neighborhood, *i.e.,* pH 5.8 to 6.0. It is certain that this optimum is considerably on the acid side of sea water, as is the case in *Arbacia* and *Asterias,* and comparatively close to the limits of acid tolerance.

The scarcity of material made it impossible to examine more closely the influence of reaction on the physical properties of the *Chætopterus* egg. It may be stated, however, that unlike *Asterias* and *Arbacia,* the *Chætopterus* egg is distinctly liquified rather than solidified at pH 4.6 to 5.0. This liquefaction is so marked that after an exposure of one or two hours the eggs are extremely fluid and will flow into thin pencils when the containing vessel is tilted or jarred. A similar liquefaction takes place from pH 9.0 to 10.0.

There is no doubt that physical changes in the nature of coagulation in acid and dispersion in alkali characterize the limits of physiological tolerance in *Arbacia* and *Asterias* eggs. But that coagulation in acid is not the invariable rule is evident from the liquefaction which occurs in *Chætopterus* eggs. It is reasonable to suppose that the specific composition of the cortex (and perhaps of the vitelline membrane as well) determines both the direction and degree of the physical changes at various reactions. The cytoplasm of all the eggs examined here is distinctly liquid (13) and it may be that the liquefaction or gelation observed is more a consequence of changes in the vitelline membrane and cortex than in the cytoplasm proper.

The general nature of the changes in physical properties and the changes accompanying ageing at various H-ion concentrations suggests that the prolongation of the life of these eggs in acid solution is a consequence of reduced metabolism. Increasing acidity up to a certain point leads, perhaps by an internal action or by a reversible alteration in the cortex which decreases the facility of interchange, to decreased metabolic activity; excessive acidity on the other hand produces irreversible injuries in the egg; where reduced metabolism and acid injury strike a reversible mean, the egg retains its viability for the longest time. The agreement between the pH at which maturation of *Asterias* eggs is completely inhibited and the pH of maximum viability conforms with this suggestion. The H-ion concentration of maximum viability may have some significance in relation to ovarian life, for in the ovary the egg is subjected to a greater CO_2 tension and H-ion concentration than that of sea water. But the cœlomic fluids of *Asterias* and *Arbacia* are approximately neutral and it is unlikely that at any time the egg would naturally be subjected to an acidity so great as pH 6.0.

SUMMARY.

A method is described for preparation of CO_2-free sea water of H-ion concentrations from pH 4.5 to 10.2.

The eggs of *Asterias, Arbacia* and *Chætopterus* retain for the longest time their capacity to fertilize and to divide at about pH 6.0.

Approximately this same H-ion concentration is required to completely repress the maturation process in *Asterias* eggs.

Chætopterus eggs are activated by exposures of 30 minutes or more to solutions of pH 5.0 to 6.6. If left in these solutions they show marked ameboid movements and fragmentation, but do not divide. If returned to sea water half or more of the eggs will undergo one or two abortive divisions. The activating effect of the acid sea water is most intense at about pH 5.8 to 6.0.

An exposure of 5 or 10 minutes to solutions of pH 5.0 to 6.6 (which is insufficient to activate) creates a block to fertilization which is permanent if the eggs are returned to sea water. If left from 30 minutes to several hours in the acid sea water, these eggs gradually recover their fertilizability, and when inseminated develop almost normally.

We are indebted to Mabel T. Studebaker for the statistical work in the experiments recorded in this paper.

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BIOLOGICAL BULLETIN

THE INFLUENCE OF HYDROGEN ION CONCENTRATION ON THE DEVELOPMENT OF NORMALLY FERTILIZED *ARBACIA* AND *ASTERIAS* EGGS.

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The belief that cessation and initiation of development in the marine egg depended in some manner on the ionic equilibria of sea water led Loeb (1) to examine the influence of changes in the concentration of H- and OH-ions on the development of normally fertilized eggs. He found that the development of the eggs of *Arbacia* is retarded and finally prevented if increasing quantities of acid are added to sea water, and that the development to the pluteus stage is accelerated in alkaline sea water. The latter fact was indicated by the advanced size and development of the plutei formed from the treated eggs as compared with controls. On subsequent investigation he concluded that alkali does not accelerate the early cleavage rate, but only the later development from the blastula to the pluteus. The addition of excessive quantities of alkali had an injurious effect. The maximum stimulation was observed when 1.75 cc. $N/10$ NaOH were added to 100 cc. sea water. He attempted to raise the newly fertilized eggs of *Strongylocentrotus* in a neutral Ringer's solution without success, but found that with the addition of a small quantity of KOH, or better $NaHCO_3$, good larvæ might be obtained. He concluded that a neutral or faintly alkaline solution is necessary for normal development (2). This conclusion was reached from other points of view by Herbst (3) and Peter (4).

Moore, Roaf and Whitley (5) performed similar experiments with the eggs of *Echinus esculentus;* the addition of small amounts of alkalies or alkaline salts, such as Na_2HPO_4, to sea water in which the eggs were growing caused an increase in the rate of growth in the early as well as the late stages, but larger amounts led to abnormal division. They pointed out that in some eggs in quite alkaline solutions nuclear division occurred without cytoplasmic division, so that the blastomeres became multi-nucleated. Still larger amounts of alkali inhibited both nuclear and cytoplasmic division. On the other hand, the smallest amount of acid had only an inhibitive action. There was no tendency for nuclear division without cytoplasmic division; and with comparatively small amounts of acid cell division was completely prevented. They concluded that the extreme limits of reaction at which cell division is possible lie very close together, and they pointed out that the phosphates and carbonates in sea water have a "steadying action" against fluctuations in the concentrations of H- and OH-ions which must be advantageous to cell growth. Subsequently, Whitley (6) found that small quantities of acid and alkali were very injurious to the developing eggs of the plaice, *Pleuronectes platessa.* No accelerating effect was observed in alkaline sea water, but Whitley concluded that a disturbance of the equilibrium towards the acid side is much more fatal than the opposite. There appeared to be an increase in resistance to unfavorable reactions developed in proportion to the age of the larvæ.

Glaser (7) repeated Loeb's experiments with *Arbacia* in another connection and concluded that accelerated development in alkaline sea water is limited to the development from the blastula to the pluteus, and that the early rate of cleavage is not accelerated, and may even be suppressed. Glaser noted the time required for the successive cleavage planes to appear in the majority of eggs in the cultures. By this method a small change in velocity of development would be difficult to detect, though it would become manifest if continued to the later stages where its results would of course be magnified.

Although it had another objective, the excellent work of Medes (8) on the causes of variation in the larvæ of *Arbacia* is of interest in this connection. Medes made careful comparative measurements on the skeletons of plutei obtained by inseminating and raising the eggs in sea water to which various substances had been added. She found that HCl, CO_2 and acetic acid markedly retarded development. In view of Loeb's statement that alkali does not have any effect on the early development of *Arbacia,* she reexamined this point by counting the number of divided eggs one hour after insemination, and by comparing the skeletal development of the larvæ 18 hours after insemination. She found a definite acceleration from one to 18 hours with NaOH (greatest in 1.33 cc. $N/10$ NaOH + 98.66 cc. sea water) and with Na_2CO_3 (greatest in 0.4 cc. 0.45 M. Na_2CO_3 + 99.6 cc. sea water), though in later stages the alkali cultures showed a retardation so that ultimately they lagged behind the controls. Larger quantities of NaOH and Na_2CO_3 inhibited development from the beginning. NaCl produced slender, perforated skeletons with conspicuous processes; there was inhibition during early development and excessive growth during later periods. NaOH led to irregularity and asymmetry, while $NaHCO_3$ increased the bulk of the skeleton with a strong tendency for regularity and symmetry.

Richards (9) has recently observed acceleration of the early cleavage rate of the eggs of the opisthobranch, *Haminea virescens.* in sea water to which NaOH and KOH had been added. No acceleration was observed after the addition of $Ba(OH)_2$ and $Cr(OH)_3$.

In none of the investigations cited were the H-ion concentrations determined or controlled, nor was allowance made for any specific influence which the carbonic acid present in acidified sea water might have. Knowing that carbonic acid inhibits cell division at H-ion concentrations which otherwise are innocuous (10), it is important to determine the limits of reaction of CO_2-free sea water within which normal development is possible.

In performing the experiments reported in this paper, *Arbacia* and *Asterias* eggs were inseminated in sea water and subsequently transferred to 100 cc. of the pH solutions prepared as described in a previous paper (11). At appropriate intervals samples of 3 to 5 cc. were removed from each lot and fixed by the addition, in the case of Arbacia, of 2 or 3 cc. of a 1–1000 solution of formalin in sea water; the *Asterias* eggs were fixed by adding 2 or 3 cc. of a 1–1000 mercury bichloride solution in sea water. These methods of fixation stop all developmental processes at once and the cleavage planes remain clearly visible for many hours. Careful counts were then made on each sample, noting the number of eggs which were undivided and the number which were in each stage of division. By multiplying the number of two-cell eggs by one, the 4-cell eggs by two, the 8-cell eggs by 3, etc., and dividing the total number of divisions by the total number of eggs, the number of divisions per egg in each sample was determined. This figure is an arithmetic index of the degree of development, or if expressed in terms of time, of the velocity of development. By counting two to 3 hundred eggs in each sample, considerable accuracy can be obtained.

A particular culture of eggs will develop under constant conditions in sea water with a mean velocity that remains practically constant so long as the number of cleavages can be accurately counted. Certain individuals will be slower than the mean and others will be faster than the mean, expressing differences in viability or developmental capacity. Such differences may be interpreted from a statistical point of view to indicate the fluctuations which any individual may undergo, and the mean to represent the behavior of the average individual. The variations observed in the development of different cultures present many interesting features which we cannot discuss at this time. It should be pointed out, however, that for studies of developmental velocity under normal and abnormal conditions, the ideal condition is to have a maximum distribution of variants ("slow" eggs and "fast" eggs) so that development will progress over short intervals of time (*i.e.,* 15 to 20 minutes) in a uniform manner. Though this condition usually obtains, there are times in the season when the eggs are in such uniform physiological condition that they divide almost simultaneously. At such times the number of divisions per egg increases by abrupt steps. This circumstance can be alleviated by averaging two successive observations on each culture. For the present purposes it will suffice to consider the mean development during the entire period of observation.

The influence of reaction on the early developmental rate of normally fertilized *Asterias* and *Arbacia* eggs is shown in Fig. 1. The data summarized in this figure are taken from several experi-

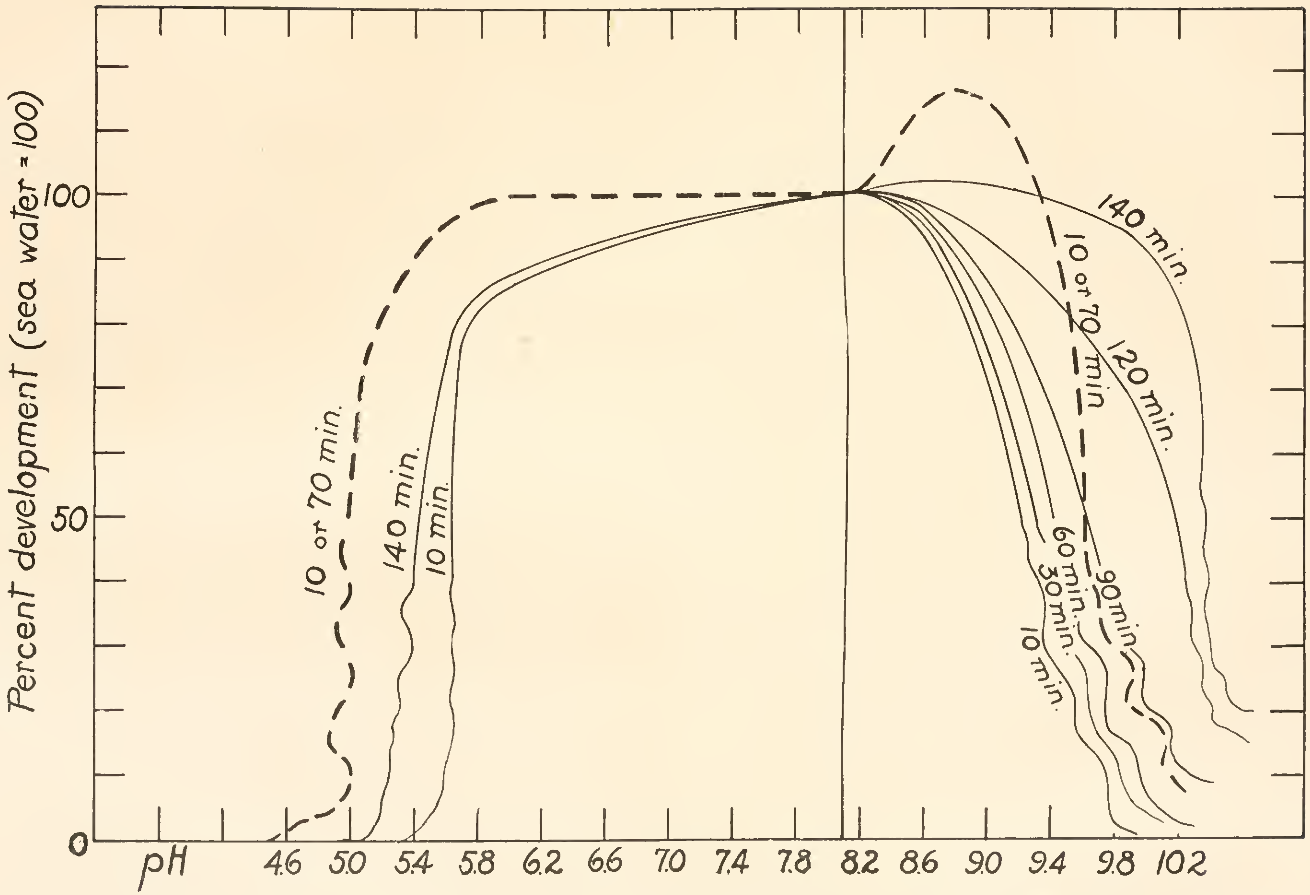

Fig. 1. The influence of H-ion concentration on the early development of normally fertilized *Arbacia* (dotted) and *Asterias* (solid line) eggs. The curves are based on the average results obtained from several experiments with each species and are expressed on the basis of the development in sea water (pH 8.15) as 100 per cent. The figures by each curve show the time after insemination at which the eggs were transferred from sea water to the acid and alkaline solutions.

ments with each species performed during the summers of 1922 and 1923. In these experiments the eggs were inseminated in sea water, centrifuged at various intervals after insemination and transferred to the pH solutions. The data given beside the curves show the time after insemination at which the eggs were transferred from sea water to the pH solutions. The development was followed quantitatively on samples taken every 20 minutes in which the number of divisions per egg was determined by careful counts. The mean development was then obtained by averaging all the observations for each solution, and the results expressed in terms of the corresponding figure for sea water as 100 per cent. The full ordinate indicates the H-ion concentration of sea water.

The dotted line in Fig. 1 refers to *Arbacia* and the solid lines to *Asterias*. The significance of the wavy portions of these lines will be discussed later. In *Arbacia* the velocity of division remains practically constant from pH 8.15 (the H-ion concentration of sea water) to pH 6.0; at 5.0 velocity of division is reduced by one half and at 4.6 division is completely suppressed. A slight increase in the alkalinity of sea water increases the velocity of division; this stimulation reaches its maximum about pH 8.8, and amounts to a 15 to 25 per cent. increase over the velocity in sea water. Above pH 8.8 there is an abrupt retardation so that the developmental velocity is reduced by one half at 9.6, and at 10.12 only a small fraction of the eggs divide even once.

Attention is called to the fact that the limiting reactions are characterized, not by a gradual, but by an abrupt inhibition of cell division within a comparatively narrow range. Between these limiting reactions cell division is essentially unimpaired.

It will be convenient for purposes of reference to define the critical limit as the pH at which the curve under consideration is reduced to its midpoint, *i.e.,* to 50 per cent. Accordingly the limits for *Arbacia* may be said to be pH 5.0 and 9.6. These limits are the same whether the eggs are placed in the pH solutions 10 minutes or 60 minutes after insemination. In the latter case, however, the degree of stimulation by alkali is slightly less.

The *Asterias* egg differs from the *Arbacia* egg notably in this—that while the resistance of the latter to both acid and alkali appears to be the same 10 minutes and 60 minutes after fertilization, the resistance of the *Asterias* egg is much lower 10 minutes after fertilization than it is later on. There is a gradual increase in resistance, particularly in alkali, from 10 minutes after fertilization until the first cleavage, at which time the maximum resistance appears to be reached. Reference to Figure 1 will show that development is inhibited at pH 9.2 if the eggs are transferred to this solution 10 minutes after fertilization; but if the eggs are not transferred until the majority have reached the two cell stage (*i.e.,* about 140 minutes) they not only tolerate pH 9.2 but they tolerate equally well pH 10.0. This difference in resistance is strikingly shown by the cultures 24 hours later. When placed, for example, in pH 9.2 10 minutes after fertilization the velocity of development is greatly reduced; in many eggs the nucleus divides without cytoplasmic division; fragmentation and abortive division are predominant and no egg progresses beyond the 32-cell stage. When placed in this same solution after the first cleavage has occurred 90 per cent. of the eggs will develop to practically normal swimmers. The increase in resistance to acid is considerably less, the limiting acidity shortly after fertilization being pH 5.6, and 140 minutes after fertilization pH 5.4. The increase in resistance to alkali which gradually appears as the egg approaches the first cleavage plane is not to be confused with the period of great susceptibility which follows the event of fertilization, or with the periodic changes in resistance to various destructive agents which a number of observers have shown to be associated with the process of cleavage. The shortest interval after fertilization at which we transferred the eggs to the pH solutions was 10 minutes, and therefore the period of great susceptibility to destructive agents immediately following fertilization was avoided. And since in our experiments the eggs are left in the pH solutions until the conclusion of the experiments, which cover in the case of *Asterias* 5 cleavages, any periodic fluctuations in resistance, if they occur, are translated into a mean.

There is little increase in the velocity of division in alkaline solution, 2 or 3 per cent. being the maximum observed in any of our experiments. There is frequently a marked stimulation shortly after transferring to the alkaline solution, but this is transient and is followed by a decrease in the velocity of division, so that after 4 or 5 cleavages the alkali cultures are about even with the controls. There is a slight but perceptible decrease of the velocity of division in acid solutions between pH 5.8 and 7.8, in contrast to the *Arbacia* egg where the velocity remains practically constant.

If we consider the limits for eggs transferred to the pH solutions at the time of the first cleavage, these limits are pH 5.4 and 10.1. Thus the limits within which the development of *Asterias* eggs is possible are distinctly on the alkaline side of those for *Arbacia* eggs.

In those solutions in which the velocity of development is reduced below 50 per cent., the quality of cell division in both *Arbacia* and *Asterias* eggs is greatly altered. The division of the cytoplasm is apparently restrained before the division of the nucleus, and in consequence the majority of eggs become multinuclear. This condition of abnormal division is indicated in Fig. 1 by the wavy portions of the curves. After two or 3 cleavages of the nucleus without cytoplasmic division the egg usually divides abruptly into more than two blastomeres, but the division is invariably abnormal and either soon ceases entirely or leads to cytolysis. In some cases it can be observed that the cytoplasm begins to divide but the furrow melts and the blastomeres fuse. The tendency for nuclear division without cytoplasmic division is much more marked in alkaline than in acid solutions. A point is reached on the alkaline side, however, where nuclear as well as cytoplasmic division is completely inhibited. A similar repression of cytoplasmic division without complete repression of nuclear division has been observed with lack of oxygen, the action of chloroform and ether, the action of hypertonic and hypotonic sea water, cold and other agents (12).

We are concerned here principally with variations in developmental velocity which are made manifest in the early history of the dividing egg, during that period of time in which accurate quantitative information can be obtained. It is of interest to consider, however, the effects of longer exposures. A method is not available for expressing these effects quantitatively but a fair idea of the degree of retardation during a 24 hour exposure can be obtained by comparing the general development of the larvæ.

Such comparisons have shown that some retardation of development occurs even at pH 7.6 and 8.5, and that the effect of increasing acidity or alkalinity does not take the form of an abrupt inhibition at any point, but manifests itself in almost imperceptible gradations from normal development to no development at all. In the acid solutions the inhibition culminates in coagulation with little division; and in alkaline solutions in either complete cytolysis or in the formation of formless, ciliated masses of protoplasm swimming within the fertilization membrane. It is doubtful if normal development can be obtained throughout a period of 24 hours in solutions more acid than pH 7.8, or more alkaline than 8.4.

SUMMARY.

The effect of acid and alkaline sea water on the rate of cell division in normally fertilized *Arbacia* and *Asterias* eggs was observed as far as the 128-cell stage.

In *Arbacia,* the velocity of division is reduced to 50 per cent. of the velocity in sea water (pH 8.15) at pH 5.2 and 9.4. Between pH 5.8 and 8.2 these eggs divide normally both in respect to velocity and quality of cell division. Between pH 8.2 and 9.2 the velocity of division is increased from 15 to 25 per cent.

Asterias eggs are more sensitive to both acid and alkaline sea water during the precleavage period than at any subsequent time. When these eggs are transferred to the acid and alkaline sea water immediately after fertilization, the velocity of division is reduced to 50 per cent. at pH 5.6 and 9.2; when transferred in the two cell stage the corresponding limits are pH 5.4 and 10.2. There is a slight decrease in the mean velocity of division between pH 8.2 and 5.8, but no significant increase in solutions more alkaline than sea water.

In both species, when the developmental velocity is reduced below 50 per cent. by either acid or alkali, the nucleus tends to divide without division of the cytoplasm, and abnormal multinuclear cells are formed.

We are indebted to Mabel T. Studebaker for the statistical work in the experiments recorded in this paper.

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THE INFLUENCE OF HYDROGEN ION CONCENTRATION ON THE FERTILIZATION PROCESS IN *ARBACIA, ASTERIAS* AND *CHÆTOPTERUS* EGGS.

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In a previous communication we pointed out that when *Asterias* and *Arbacia* eggs were inseminated in CO_2-free sea water of varying H-ion concentration, fertilization failed to occur in solutions more acid than pH 6.6 to 7.0. This block to fertilization appeared to be perfectly reversible, since eggs which did not fertilize in solutions on the acid side of the block could be fertilized when returned to solutions of greater alkalinity (1). Loeb (2) has observed a similar block to fertilization in artificial salt solutions. He found that *Arbacia* and *S. purpuratus* eggs were not fertilized in a neutral mixture of $NaCl + MgCl_2$ in the proportion in which these salts exist in sea water. These eggs were fertilized, however, if NaOH, NH_4OH, benzylamine, butylamine or $NaHCO_3$ were added to the $NaCl + MgCl_2$ mixture. The addition of $CaCl_2$ to the $NaCl + MgCl_2$ mixture similarly made fertilization possible. The addition of NaOH or $CaCl_2$ to a $NaCl + KCl$ mixture did not permit fertilization of all eggs, but when both NaOH and $CaCl_2$ were added to a $NaCl + KCl$ mixture as a rule all the eggs fertilized and began to divide. Since cross fertilization can be effected between *Asterias* sperm and *S. purpuratus* eggs by the addition of NaOH or $CaCl_2$ to normal sea water, Loeb concluded that the act of diminishing the alkalinity of the solution or of depriving it of $CaCl_2$ established the same reversible block to the entrance of the homologous sperm as exists for the entrance of the sperm of *Asterias* into *S. purpuratus* eggs in normal sea water. Leob's experiments involve the change of several variables at once, however, and it cannot be determined from them to what extent the reaction of the external medium *per se* influences the fertilization of eggs by sperm of the same species.

Further examination of the block to fertilization which is created when the H-ion concentration of sea water is increased to a critical point has convinced us of its physiological significance, and we have extended our observations to include the effects of increasing alkalinity on the fertilization of *Arbacia* and *Asterias* eggs, and the effects of acid and alkaline sea water on the fertilization of the eggs of *Chætopterus pergamentaceous.*

The Acid Block to Fertilization.

CO_2-free sea water solutions were prepared as described in a previous paper (3). Our experiments on fertilization were performed as follows: A drop of concentrated egg suspension was added to 50 or 100 cc. of each of the pH solutions, and a drop of sperm suspension was added to about 5 cc. of the pH solutions. After an interval of 3 to 5 minutes the sperm and eggs were mixed and thoroughly agitated. (No precautions were taken to remove body fluids which might be present around the eggs, other than the routine washing which they were always given in preparing them for any experiment.) Subsequently the proportion of fertilized eggs in each dish was carefully determined. It makes no difference whether the counts are made 10 minutes or several hours after insemination because every egg that is going to fertilize will lift a membrane within the normal time of 3 to 5 minutes. It has been our custom in performing experiments of this kind to remove samples from the pH solutions 10 or 15 minutes after insemination and return them to sea water with fresh sperm to make sure that the eggs had not been irreversibly modified by the action of the pH solutions or by contact with sperm in these solutions. It may be said that this procedure has one invariable result; if the exposure is below that required for the acid to injure the egg, then every egg which is not fertilized on the acid side of the block will fertilize when returned to sea water with fresh sperm.

The influence of H-ion concentration on the fertilization of *Arbacia* and *Asterias* is illustrated in Figs. 1 and 2. The solid line in each figure indicates the range within which fertilization

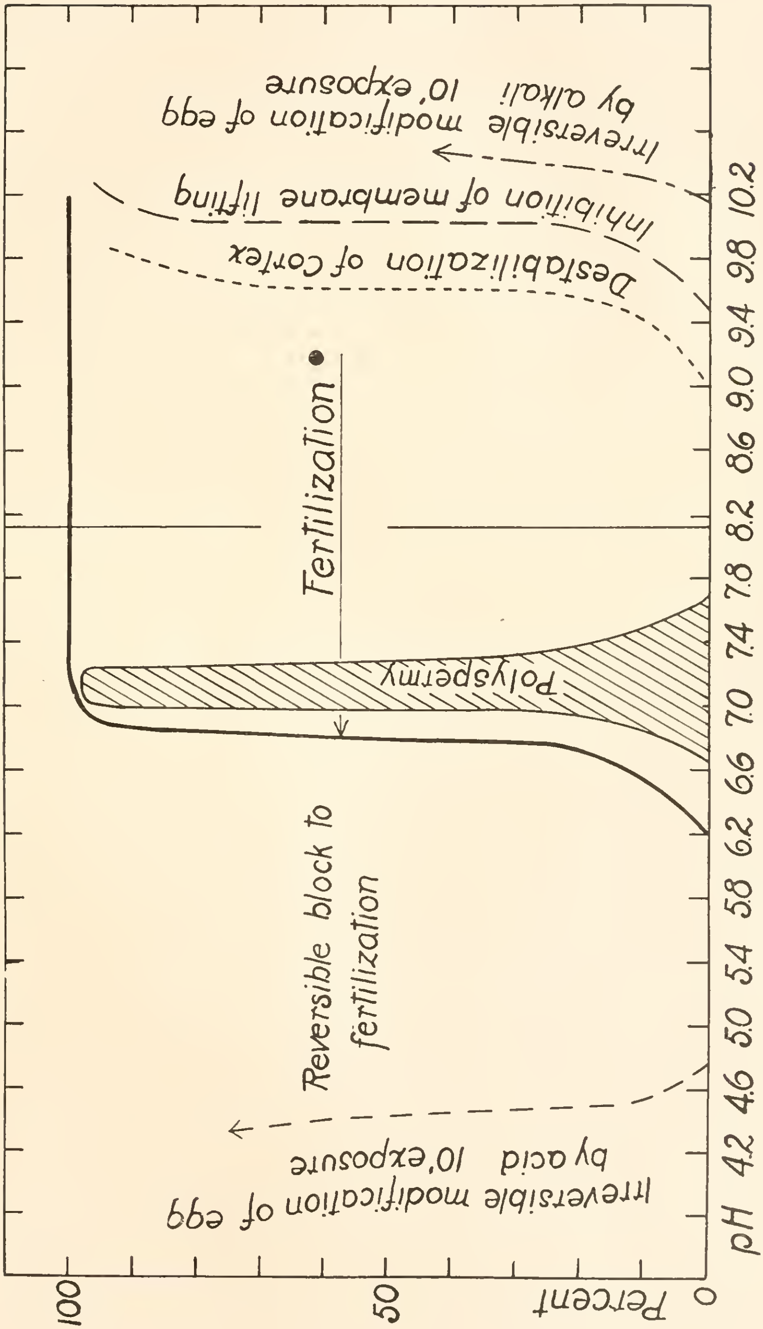

FIG. 1. The influence of H-ion concentration on the fertilization of *Arbacia* eggs.

occurs. The cessation of fertilization with increasing acidity is very abrupt, but the critical H-ion concentration may be most accurately indicated by the pH at which only 50 per cent. of the eggs fertilize. This critical H-ion concentration is pH 6.8 for *Arbacia* eggs, and 7.0 for *Asterias* eggs. In solutions slightly more alkaline than these, all the eggs fertilize; and in solutions slightly more

TABLE I.

A. ACID BLOCK TO FERTILIZATION IN *Asterias*.

Per Cent. of Eggs Fertilized.

pH									Date.
6.6	6.7	6.8	6.9	7.0	7.1	7.2	7.3	7.4	
0		0		50		100		100	Conclusions 1921.
0		1		42		100			6-30-22
0		0		21		85			7- 6-22
0		0		0		4		76	5-29-23
0		0	1	5	5	40		72	5-30-23
0	0	0	0	47	60	85		93	8-31-23
0	0	0	0	0		82		100	8-31-23
0	0	0	0	60		100		100	8-31-23
0	0	0	10	6		21		72	9- 2-23
0	0	0	14	11		22		90	9- 3-23
0	0	0	40	40		100		100	9- 3-23

B. ACID BLOCK TO FERTILIZATION IN *Arbacia*.

Per Cent. of Eggs Fertilized.

pH									Date.
6.6	6.7	6.8	6.9	7.0	7.1	7.2	7.3	7.4	
0		50		100		100		100	Results of 1921.
2		47		98		98		97	6-30-22
0	0	100	100	100	100	100		100	7-20-23
0	0	30	20	75	100				9-21-23
2	7	28	24	95	100	100	100		9-22-23
7	29	100	100	100	100	100	100	100	9-22-23
0	0	30	20	75	100	100	100	100	9-22-23

C. COMPARISON OF ACID BLOCK IN *Asterias* AND *Arbacia* IN SAME pH SOLUTIONS. 9-21-23.

Per Cent. of Eggs Fertilized.

	pH								
	6.6	6.7	6.8	6.9	7.0	7.1	7.2	7.3	7.4
Asterias.	0	0	0	1	30	100	100	100	100
Arbacia.	5	0	30	70	100	100	100	100	100

acid, none of the eggs fertilize. We have previously shown that the unfertilized eggs of these species are uninjured by short exposures to the solutions in which fertilization does not occur (3); and that normally fertilized eggs will develop with normal velocity at H-ion concentrations much greater than those at which the block to fertilization occurs (4).

The constancy of this acid block to fertilization is very marked. To illustrate this a few experiments have been given below. Table I. contains a résumé of experiments performed in 1921, 1922 and 1923 on the fertilization of *Asterias* and *Arbacia* eggs. Despite the probability of variable conditions in these experiments, the point at which 50 per cent. of the eggs fertilized remained constant to ± 0.2 pH.

There are at least two factors which might be expected to shift the block one direction or another; first, the length of time which the eggs or sperm have remained in the acid solution, and second, the relative quantity of sperm used for insemination. Examination of the first factor has shown that the equilibrium between the pH solution and the egg (or sperm) is reached with astonishing rapidity. This can be illustrated by first adding the sperm to the pH solution and then adding to the resulting sperm suspension a drop of eggs suspended in sea water. Under these conditions one would expect that the time required for the egg cortex to come to chemical equilibrium with the pH solution would be long enough to permit many more eggs to be reached by sperm and fertilized than would be the case if the eggs were allowed to come to equilibrium with the solution before adding the sperm. The results of experiments of this kind with *Asterias* eggs are given in Table II. Converse experiments were simultaneously performed; the eggs were added to the pH solution first and after 5 minutes a drop of comparatively concentrated sperm suspended in sea water was added to these eggs. When the experiment is performed as first described, the block appears at the same pH as when both eggs and sperm are at equilibrium with the pH solutions before insemination. When the experiment is reversed, the block is shifted slightly towards the alkaline side. This indicates that the essential equilibrium underlying the block involves the egg cortex rather than the sperm. The difference is hardly great enough to be significant, though the results do show very definitely that chemical equilibrium between either eggs or sperm and the pH solutions is reached in less time than is required for sperm to reach the eggs and fertilize them.

Table II.

A. Effect of Exposing *Asterias* Sperm to pH Solutions for 5 Minutes before Adding *Asterias* Eggs in Sea Water. 8–31–23.

Per Cent. Eggs Fertilized.

pH									
6.2	6.6	6.8	6.9	7.0	7.1	7.2	7.6	8.15	
0	0	0	6	8	81	85	90	83	(Exp. 1)
0	0	0	0	47	85	90	89	97	(Exp. 2)

B. Effect of Exposing *Asterias* Eggs to pH Solutions for 5 Minutes before Adding *Asterias* Sperm in Sea Water. 8–31–23.

Per Cent. Eggs Fertilized.

pH								
6.2	6.6	6.8	6.9	7.0	7.1	7.2	7.6	
0	0	0	0	0	49	82	100	(Exp. 1)
0	0	0	0	2	63	95	100	(Exp. 2)

One of several experiments testing the influence of varying quantities of sperm in shifting the limits of fertilization is given in Table III. The eggs were placed in the pH solutions and 5 minutes later the sperm, diluted with the pH solutions, were added.

Table III.

Influence of Quantity of Sperm on Acid Block in *Asterias*.

Per Cent. Eggs Fertilized.

pH										Quantity of sperm added to 25 cc. pH sol.
6.6	6.7	6.8	6.9	7.0	7.1	7.2	7.3	7.4	8.15	
0	0	0	50	100	95	95	100	100	100	1 cc. 1–20
0	0	0	0	35	80	95	100	100	100	1 cc. 1–200
0	0	0	0	0	20	85	87	95	90	1 cc. 1–2,000
0	0	0	0	1	40	45	25	35	30	1 cc. 1–20,000

The smallest quantity of sperm was insufficient to fertilize all the eggs even in sea water, and the largest quantity gave a distinctly opalescent suspension; yet the block did not shift beyond the limits pH 6.9 to 7.1. In general, increasing the quantity of sperm used in insemination increases the proportion of eggs fertilized in the acid solutions, but the shift to the acid side is not so great as would be expected if the failure to fertilize in the acid solutions were attributable to an impairment of the sperm. Rather the slight magnitude of this shift favors the belief that the block is due to an alteration of the properties of the egg.

It may be stated here that unless the sperm are injured or attenuated by the toxic action of egg secretions, all eggs which fertilize in the pH solutions develop normally, indicating that the fertilization reaction when once initiated in the neighborhood of the block, is completed without impairment.

The Acid Block in *Chætopterus*.

The determination of the acid block to fertilization in *Chætopterus* was made in the same manner as in *Asterias* and *Arbacia*. The egg sacks were cut in sea water and the eggs liberated from the ovaries by teasing these to pieces. The ovary fragments were removed by straining through cheese cloth, and when the eggs had maturated they were concentrated by centrifuging. A drop of the concentrated egg suspension was added to 50 cc. of the pH solution; after 5 minutes the sperm, previously diluted with the pH solutions, were added and the mixture agitated. The per cent. of fertilized eggs was determined by counting the dividing eggs one and a half to four hours after insemination.

The scarcity of material made it impossible to get more than a half dozen determinations; of these, two were discarded since only a small proportion of the eggs were fertilized in sea water. The remaining four indicated that the block appeared between pH 7.0 and 7.3, and from the two most satisfactory experiments the block was tentatively set at pH 7.1.

The acid (pH 5.8) activation of the *Chætopterus* egg, with the consequent temporary block to fertilization, has been discussed in a previous paper (3). This block, which is most effectually established by short exposures to pH 5.2 to 6.4, was tentatively ascribed to cortical changes which tend to persist after the eggs have been removed from the acid solutions, and returned to sea water. It is in no sense comparable to the physiological block occurring at pH 7.1; the latter is perfectly and instantly reversible, as in *Asterias* and *Arbacia,* disappearing as soon as the eggs are returned to a more alkaline solution.

The Influence of Alkali on the Fertilization Reaction.

Frank Lillie (5) has observed that the addition of alkali to the sea water in which insemination occurs increases the incidence of fertilization in *Asterias* and *Arbacia* eggs of poor quality. We have confirmed this in *Asterias* and *Arbacia* and found that it is equally true for *Chætopterus.* This effect of a slight increase in alkalinity in aiding fertilization may be due to action on the sperm but it seems more probable that both the eggs and the sperm are affected. The changes which culminate in increased fluidity of the egg cortex in alkaline sea water (3) are no doubt preceded by enhanced physiological reactivity.

Apart from this stimulating action of alkali, which is not apparent in eggs of the best quality, fertilization in *Asterias* and *Arbacia* proceeds unimpaired from pH 8.15 to 9.6. With further increases in alkalinity, eggs appear in increasing numbers which have either tight or incompletely formed fertilization membranes; and at pH 10.2 the eggs have no demonstrable membranes at all. When returned to sea water after a 3 to 5 minute exposure to pH 10.0, fertilization membranes will form on most of the previously unmembraned eggs. It was concluded that these eggs were fertilized while in the alkaline solution, since the supernatant sperm carried over from the alkaline solution are incapable of fertilizing fresh eggs. Longer exposures injure the eggs to such an extent that membranes do not form on them when they are returned to sea water. The H-ion concentrations which prevent membrane elevation (and which destabilize the cortex of the unfertilized egg (4)) are shown in Figs. 1 and 2 by the dotted lines at the extreme right.

This alkaline injury is more rapid in *Asterias* than in *Arbacia.* In the latter case the eggs will divide imperfectly if returned to sea water after a 5 to 10 minute exposure to the alkaline solution. In *Asterias* the inhibition of membrane elevation is rapidly followed by a more profound injury which completely stops development. Such eggs can not be fertilized by fresh sperm in sea water.

These facts all indicate that under increased alkalinity union of the egg and sperm still occurs; but if the increased alkalinity

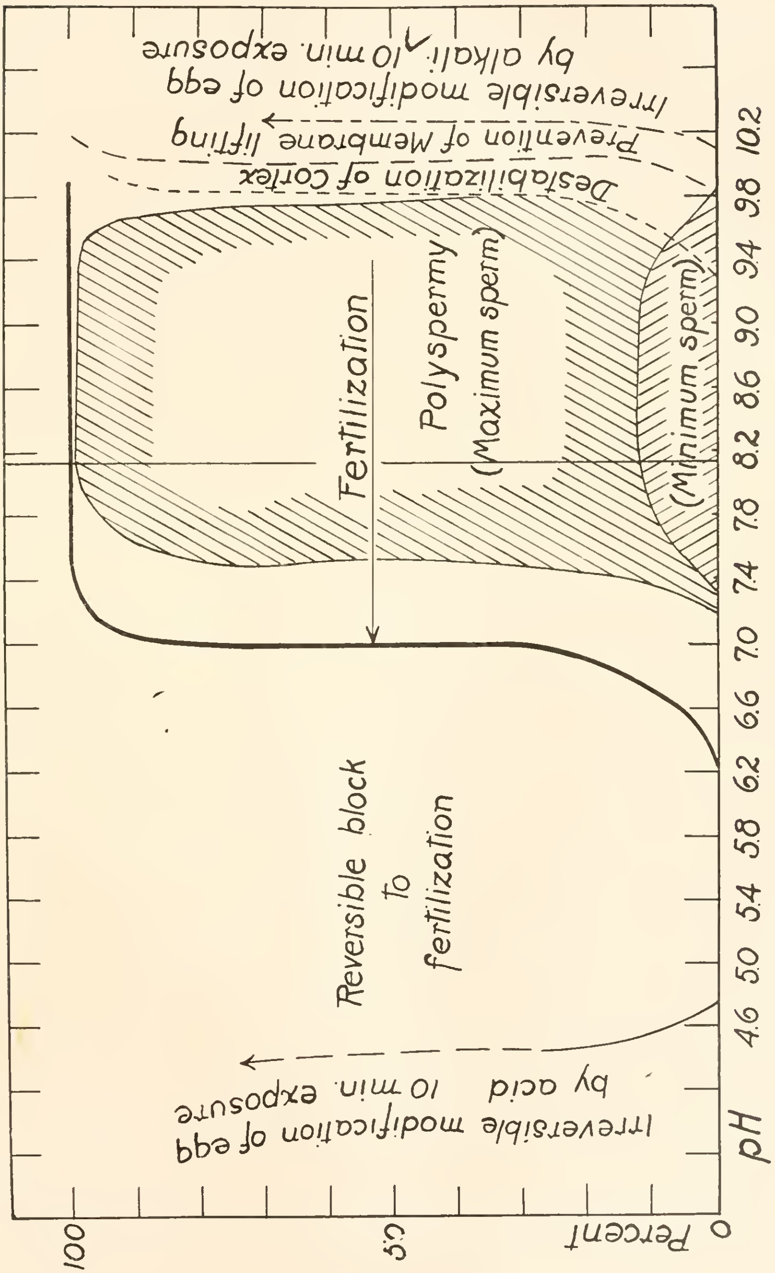

FIG. 2. The influence of H-ion concentration on the fertilization of *Asterias* eggs.

does not actually introduce some abnormality into this initial event, it impairs subsequent events of the fertilization process to such a degree as to prevent normal development. There is apparently no reversible block created by alkali corresponding to that created by acid, where the fertilization reaction proceeds in an all-or-none fashion. This conclusion is supported by the extremely rapid injury of the eggs and sperm if separately exposed to the alkaline solutions (pH 10.0), which prevent normal fertilization, and the complete absence of such injury in acid solutions (pH 6.8 to 7.0) which have a similar effect.

POLYSPERMY.

In the case of *Arbacia* eggs there is a very narrow range in H-ion concentration in which the incidence of polyspermy is unusually high. This range is approximately defined in Figure 1 by the heavily shaded portion; the maximum of polyspermy is close to pH 7.2. Though the incidence of polyspermy at all H-ion concentrations increases with increasing age or staleing of the eggs, yet within this narrow range, centering at pH 7.2, practically all the eggs will be polyspermic even when they are fresh and when the incidence of polyspermy is nearly zero from pH 7.4 to 9.8.

In *Asterias,* polyspermy shows no marked maximum at any H-ion concentration but occurs more or less uniformly from pH 8.5 to 9.5 (Fig. 2). When excessive quantities of sperm are used in insemination, nearly all the eggs may be polyspermic from 8.15 to 9.6. It is perhaps significant that the polyspermy curve, even though extremely broad, is limited on the alkaline side; for the incidence of polyspermy decreases appreciably before the alkalinity is sufficient to inhibit fertilization, indicating that in its general nature the underlying mechanism in *Asterias* is similar to that in *Arbacia.*

We did not have the opportunity to make similar observations on polyspermy in *Chætopterus.* Such data as we have indicate that there is, as in *Arbacia,* a comparatively narrow region in which polyspermy predominates (about pH 9.5).

Reaction of Sperm with Immature *Asterias* Eggs.

When immature *Asterias* eggs are inseminated in sea water, several sperm usually enter each egg before the fertilization membrane is formed. Subsequently the germinal vesicle breaks down and the cytoplasm acquires a mottled appearance, each sperm being the focus of a localized cytolytic process. Such prematurely fertilized eggs never attempt to divide. If sperm are added to immature eggs at various H-ion concentrations, a block appears at the same point as in the fertilization of the mature egg, viz., pH 7.0. On the alkaline side of this point the sperm enter the eggs, causing membrane elevation and the changes described above. On the acid side the sperm do not react with the eggs in any way; in the course of time, a varying proportion of these unfertilized eggs will maturate, depending on the H-ion concentration, and these, if they are returned to sea water and inseminated, will fertilize and develop normally.

Summary.

When *Arbacia, Asterias* and *Chætopterus* eggs are inseminated in CO_2-free sea water of varying H-ion concentration, a block to fertilization appears at a H-ion concentration which is constant, and apparently characteristic for each species. If the block is defined by the H-ion concentration at which 50 per cent. of the eggs fertilize, these H-ion concentrations are: *Arbacia,* pH 6.8; *Asterias,* pH 7.0; and *Chætopterus,* pH 7.1.

This block to fertilization is complete, in that eggs either fertilize and develop normally, or do not fertilize at all; and it is perfectly reversible, in that eggs which do not fertilize on the acid side of the block will fertilize immediately if they are returned to solutions on the alkaline side of the block and inseminated with fresh sperm.

In sea water more alkaline than pH 9.8 to 10.0 the fertilization process in both *Arbacia* and *Asterias* eggs is either incomplete or impaired. Apparently there is no alkaline block to fertilization corresponding in its complete reversibility to the block which appears around neutrality.

In *Arbacia* there is an increased incidence of polyspermy within a very narrow range centering at pH 7.2, indicating some critical condition in the mechanism of fertilization at this H-ion concentration. In *Asterias* polyspermy occurs more or less uniformly over a wide range extending from pH 7.2 to 9.8.

We are indebted to Mabel T. Studebaker for the statistical work in the experiments recorded in this paper.

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HERMAPHRODITISM IN *EURYCEA BISLINEATA*.[1]

INEZ WHIPPLE WILDER AND ELIZABETH BARRETT PEABODY.

The occurrence of hermaphroditism among anurans seems to be an accepted fact. Crew ('21) summarized all the recorded cases of abnormal sexual organs in frogs and states that there are forty such cases. To this Swingle ('22) has recently added one more, but finds that of his list of forty-one abnormalties only twenty-seven can be considered hermaphrodites, a sufficient number, however, coming from the hands of so severe a critic, to warrant the statement that hermaphroditism in anurans does occur. Cerruti ('07) and King ('10) following numerous earlier writers, have investigated the occurrence of the anomaly in toads with results which, though possibly subject to differences in interpretation, tend nevertheless to substantiate the existence of hermaphroditism in these forms.

No one has done for the urodeles the service which Crew has performed for the frogs, but from the paucity of published reports upon anomalies in urodeles this would not seem to be an arduous task. Thus Chapin ('15) in reporting a case of hermaphroditism found by her in *Spelerpes bislineatus* (*Eurycea bislineata*) cited reports of only two other cases of this anomaly in urodeles which had come to her attention, one that of La Valette St. George ('95) in *Triton tæniatus*, the other that of Knappe ('86) in a young *Salamandra maculata*. Since the publication of Chapin's paper a third case has been reported by Krizenecky ('17) in *Triton cristatus*. Although the cases of La Valette St. George and of Křizenecky in *Triton* are unquestionably to be accepted as genuine, there is doubt concerning the nature of the anomaly reported by Knappe in *Salamandra*. Its interpretation as an hermaphrodite is apparently that of King ('10) who in summing up the reported cases of the occurrence of hermaphroditism in urodeles says that "Knappe ('86) noted the presence of a Bidder's organ in a young salamander." In the paper in question, however, following the enumeration of the species of Amphibia which he had examined for the possible occurrence of a Bidder's organ, an enumeration which included seven species of *Anura* and two of *Urodelæ* (*Triton tæniatus* and *Salamandra maculata*) Knappe states definitely "Bald stellte sich heraus, dass es zur Bildung eines Bidder'schen Organs nur bei den echten Krötenarten kommt." And farther on after mentioning the unique appearance of one Bidder's organ, he says: "Eine solche Samenkörperbildung in Eikapseln des Bidder'schen Organs mit gleiches Bestimmtheit nachzuweisen, wie in dem eben beschriebenen Falle, ist mir bis jetzt nicht wieder gelungen, doch kann ich ähnliches für eine andere verwandte Thiergruppe, die Salamander, konstatiren. So liess eine in Schnittserien zerlegte Hodenabtheilung eines jungen, viellicht zweijährigen Salamandra maculata nicht den geringsten Zweifel, dass dieselbe aus Eikapseln, ähnlich denen im Bidder'schen Organ der Kröten, bestäht." It was thus obviously not Knappe's intention to state that he found a Bidder's organ in a salamander, but rather an appearance in the testis of a salamander like that of the unique Bidder's organ in a toad. In any case the interpretation of the condition described in the salamander as hermaphroditic, will depend upon the interpretation of the sexual nature of Bidder's organ itself. This is a matter which has been a bone of contention ever since the discovery of the organ in 1758 by Rösel von Rosenhof, and a number of theories have been advanced regarding its nature and significance.

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[1] Contribution from the Department of Zoölogy of Smith College, No. 116.

In view of the almost universal agreement of modern writers as to the femaleness of Bidder's organ, Swingle's recent discussion ('21 and '22) of its nature is of great importance. In a discussion of the so-called transformation of sex in frogs, he claims that the theory is really based on a misinterpretation of the appearance of the cells in the Bidder's organ of toads. According to Swingle, the oviform-like cells of this organ do not represent the cells of an ovary, thus making the animal an hermaphrodite at this stage, but are, like the cells of similar appearance which occur in the protestis of the frogs, merely senescent male cells which are undergoing oviform degeneration. He adds further: "True hermaphroditism in frogs is a permanent and pathological condition, probably due to a mix-up in the genetic constitution of the individual.

and is not to be confused with the present problem which has to do with a normal but transitory embryological process."

The general opinion thus set forth by Swingle finds support also in a statement made by Crew ('21) who said: "Cytologically it has not been proved that the cells which constitute Bidder's organ are ovarian and there undoubtedly are reasons for questioning the generally accepted opinion that this organ is a rudimentary ovary."

The three cases of hermaphroditism already reported in urodeles, disregarding now Knappe's inconclusive report, differ from each other quite markedly. La Valette St. George's case in *Triton tæniatus* was referred to by Cole ('96) as "the most complete case of hermaphroditism yet recorded among the Amphibia." The specimen was a male with perfectly distinct and independent paired ovaries, in addition to a pair of normal testes, but, however, without any traces of oviducts. The testes contained developing and fully developed sperms; and the ovaries, eggs in various stages of maturity.

The case reported by Křizenecky in *Triton cristatus* showed the presence of ova within both peripheral and internal lobules of otherwise normal testes.

The case reported by Chapin in *Eurycea bislineata* was that of an advanced larva in which the gonad was essentially male with female elements. Macroscopically, the anterior part of the left gonad, which was much reduced in size, resembled the normal testis in texture, though not in shape, while the posterior region was distinctly like an ovary. The right gonad, which was somewhat smaller than the normal testis of an individual of the same size, showed another sort of hermaphroditism. Two ova were found in the otherwise apparently normal testis, each one completely filling one lobule, which would normally contain a large number of male cells. This case showed, therefore, two ways in which female elements may be disposed in otherwise distinctly male gonads; one in the form of growing ova among the cysts of spermatogonia, and the other by a modification of a part of the gonad into a region resembling an ovary. The numerous cases of hermaphroditism which we have found in this same species are all of the same general character as that described by Chapin.

In the light of a recent article by Jordan ('22), it may be well to define our use of the term, hermaphroditism. According to Jordan, true anatomic hermaphroditism occurs " where ovary and testis are present in the same individual." Jordan regards the presence of an ovo-testis as a modification of true hermaphroditism, a condition which he designates as a type of false hermaphroditism. Were this distinction to be accepted, the term, true hermaphroditism, could be used only when referring to such a case as that of La Valette St. George's in *Triton tæniatus.* There seems to be no justification, however, for this distinction of Jordan's, inasmuch as a distinct testis and ovary is but a further step in the separation of the male and female elements which, in some individuals, are still intermingled to a greater or less extent in the ovo-testis. An examination of the adult ovo-testis shown in

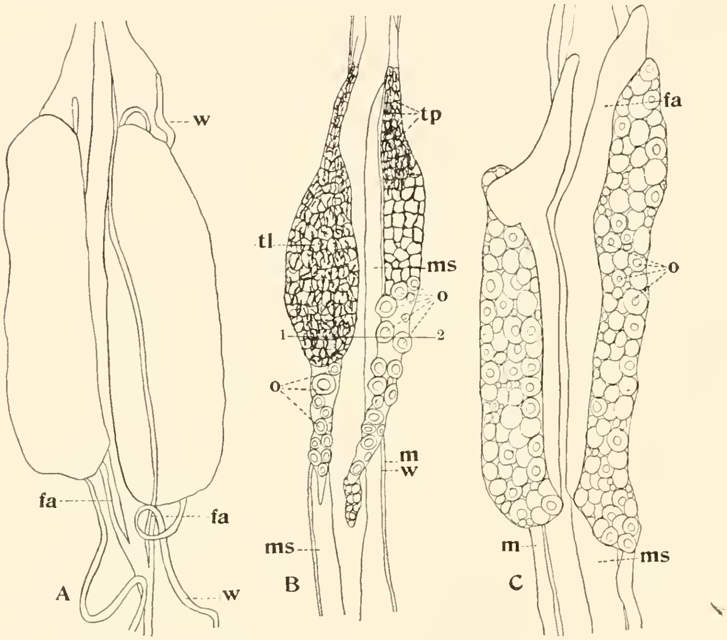

FIG. 1. Camera lucida drawings of the ventral view of the gonads of (*A*) an adult male; (*B*) an adult hermaphrodite; and (*C*) an adult female (× 9). *Fa,* fat bodies; *m,* Müllerian duct; *ms,* mesonephros; *o,* ova (primary oöcytes). *tl,* testicular lobules; *tp,* testicular pigmentation (the two latter present but not shown in (*A*)); *w,* Wolffian duct. The line 1–2 shows the level of the section drawn in Fig. 4.

Fig. 1 *B* shows that a separation posterior to the testicular part on each side, such as, in fact, is slightly indicated in the right gonad, would transform each ovo-testis into a distinct testis and ovary. A female was found in which the ovary (Fig. 2) showed a number of separate parts or lobes, some connected with each other by the mesovarium and others quite distinct, serving to illustrate the point that parts which are usually continuous may, through some unknown cause, become thus carried apart. Jordan's distinction in terminology seems, therefore, a somewhat arbitrary one, at least as applied to our species, and thus any individual which shows the presence of both male and female sex cells, even though these appear side by side in the same gonad, is regarded in this paper as a true hermaphrodite.

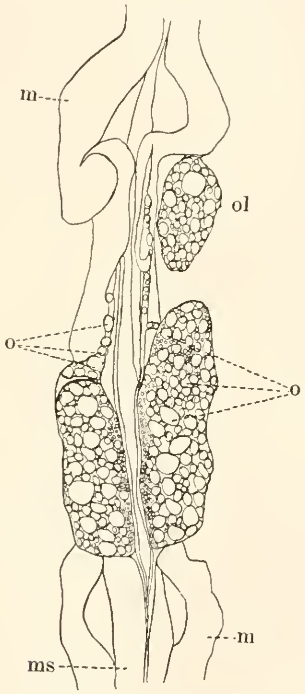

Fig. 2. Camera lucida drawing of the ovaries of an adult female showing an unusual lobed form (× 7). *M,* Müllerian duct; *ms,* mesonephros; *o,* ova; *ol,* detached lobe of ovary.

Naturally the ultimate criterion of hermaphroditism should be the production of functional germ cells of both sexes. Such a criterion would obviate all possibility of the condition being given the interpretation which Swingle has given to the oviform cells which occur in Bidder's organ and in the larval testis (pro-testis) of frogs. In carrying out our investigation we had in mind as near an approach to this ideal as possible, and, having found examples of the condition which to us seemed unquestionably hermaphroditic in individuals of various stages up to transformation, we made a definite search for such cases among adult animals. As this search was rewarded by the discovery of one adult in which the hermaphroditic condition was beyond question, although the individual had not arrived at full sexual maturity, we feel confident that our interpretation of our cases as true hermaphrodites is correct and that the condition described cannot be considered as "a normal but transitory embryological process."

Percentage of Occurrence.

The determination of the percentage of occurrence of hermaphrodites with reference to that of males and females in *Eurycea bislineata* is based upon the examination of the gonads of 1113 individuals ranging from the typical larval to the adult stage. Wilder ('24) has shown that *Eurycea bislineata* is a form in which the period before transformation is considerably prolonged, covering from two to three years, although the structural changes leading toward metamorphosis are inaugurated many months previous to the actual transformation. The whole period from hatching to transformation is subdivided on the basis of structural changes into stages, the readily recognizable criteria of which, in living individuals, are as follows:

1. Postembryonic stage—Yolk still present, intestine not fully formed.
2. Typical larval stage—Intestine fully formed; no naso-lacrimal groove and no *os thyreoideum*.
3. Premetamorphic stage—Open naso-lacrimal groove (in incipient phase); *os thyreoideum* present; no vesicular glands in the skin.
4. Metamorphic stage—Glands of skin appearing as tiny acinous vesicles (in incipient phase), becoming rapidly larger and more conspicuous; absorption of larval structures and de-

velopment of eyelids and naso-labial groove (advanced phase).

The specimens used constituted representative collections made through a period of several years and had been preserved either in alcohol after fixation in Bouin's solution, or in formalin.

The method followed in sexing was first to examine the gonads *in situ* under a Bausch and Lomb binocular dissecting microscope with a strong artificial illumination. In many cases this was sufficient to diagnose the sex, but in those cases in which it was not, the gonad was removed and cleared *in toto* in glycerine for more careful study under the compound microscope. If in sexing one begins with adults and continues through the smaller and earlier stages, one comes finally to a point where it is practically impossible to be sure of the sex. Individuals of less than 27 mm., though frequently possessing readily sexed gonads, more often exhibit a developmental condition which might admit of various interpretations, since, at least without the use of cytological criteria, the small cells present might be either oögonia or spermatogonia. Bouin ('01) found that in *Rana temporaria* the first development of male and female germ-cells is identical as far as origin and general appearance are concerned. It may even be the case in *Eurycea,* as Okkelberg ('21) has shown for the brook lamprey, that the animal passes through a period of sex indifference before sex differentiation sets in. His observations " seem to warrant the conclusion that each larva of this species (*Entosphenus wilderi*) carries the potentiality of both sexes and that sex, therefore, is not irrevocably fixed at fertilization." He explains the development of sex in these gonads of " potentially either " sex by showing the presence in the gland of two kinds of germ cells, those manifesting a tendency towards rapid division (katabolic) and those showing a tendency to growth (anabolic). He says: " The former are regarded as having a male and the latter a female potentiality. The relative proportion of anabolic and katabolic cells determines whether the larva becomes a male or a female individual."

Since our problem, however, was not one dealing with a possible early transitory hermaphroditic condition, we have included in the calculation of the percentage of occurrence only those individuals which had definitely passed beyond the indeterminate sexual stage to a point where sex could be definitely diagnosed. The following summary is, therefore, based upon the study of gonads of animals of a length of 27 mm. and over, the arbitrary minimum of 27 mm. being taken as approximately representing the dividing line between those individuals in which the sex is still, if not indifferent, at least frequently difficult to determine, and those in which the sex is unquestionably established and recognizable.

Summary of Percentage of Occurrence.

Typical larval individuals (of 27 mm. and over in length)	
Total number sexed	178
Number of males	86, or 48.3 %
Number of females	89, or 50.0 %
Number of hermaphrodites	3, or 1.68%
Individuals in incipient phase of premetamorphosis	
Total number sexed	333
Number of males	177, or 53.2 %
Number of females	152, or 45.7 %
Number of hermaphrodites	4, or 1.2 %
Premetamorphic individuals (beyond incipient phase)	
Total number sexed	256
Number of males	119, or 46.5 %
Number of females	134, or 52.3 %
Number of hermaphrodites	3, or 1.2 %
Metamorphic individuals	
Total number sexed	226
Number of males	115, or 50.8 %
Number of females	107, or 47.4 %
Number of hermaphrodites	4, or 1.8 %
Adult individuals	
Total number sexed	120
Number of males	77, or 64.0 %
Number of females	42, or 35.0 %
Number of hermaphrodites	1, or 0.83%
All individuals exclusive of adults	
Total number sexed	993
Number of males	497, or 50.05%
Number of females	482, or 48.53%
Number of hermaphrodites	14, or 1.41%

All individuals

Total number sexed	1113
Number of males	574, or 51.57%
Number of females	524, or 47.08%
Number of hermaphrodites	15, or 1.35%

The variation in the percentage of occurrence of hermaphrodites in the different developmental stages has little significance because of the large probable error due to the small number of specimens examined. It should be noted that the nearest approximation to the general average, 1.35 per cent., occurs in the case of those stages in which there were the largest numbers examined. The extremely low percentage in the adult group is noteworthy and, though probably due to the small number of specimens used, may conceivably indicate a lower degree of viability in the case of hermaphrodites in adult life. The discrepancy in percentage of males and females in the adult group may also indicate a difference in viability, but is more likely to be due to the fact that the collections used were made mainly in the spring when the females would be more difficult to find because during the egg laying period they are under large rocks in the deeper water.

The fact of real significance is that of the existence of hermaphroditism in every developmental stage, since together with the approximate equality of the two sexes, it serves to eliminate any claim that the condition in question is, in this species, merely a transitory one.

This establishment of a fairly constant percentage of occurrence of hermaphroditism in *Eurycea bislineata* suggests the possibility that a search for the phenomenon in other urodeles might reveal a like frequency of occurrence. The urodeles offer an inviting field for such investigation since so little has been done with them in connection with the problem in contrast to the large amount of attention which has been given to the anurans.

Description of Representative Cases of Hermaphroditism in *Eurycea bislineata*.

Adult Stage.

The one hermaphroditic adult found was an individual of 65 mm. in length which had the external characteristics of a female, as shown by the presence of a spermatheca in the dorsal wall of the cloaca, and the shape of the head. The appearance of the gonads *in situ* is shown in Fig. 1 *B*. For comparison, a typical ovary and a typical testis from animals of approximately the same size and collected at the same time (July 3, 1915) are shown in Figs. 1 *A* and 1 *C*. The smaller size of both testicular and ovarian parts of the ovo-testis as compared with the typical male and female gonad respectively will be noted. Moreover, while the reproductive ducts (Wolffian and Müllerian respectively) of the normal male and female have approximately the form characteristic of the mature condition, the hermaphrodite showed macroscopically apparently a single slender straight duct upon each side following the lateral border of the mesonephros. Subsequent microscopic examinations of cross sections of this, however, revealed the presence of two ducts (cf. Fig. 3) the more lateral of which, the Müllerian, alone persists anterior to the mesonephros. This sexually indifferent condition of the ducts is identical with that shown by cross sections, made previous to this investigation, through the body of a 66 mm. immature adult male, the testes of which also correspond histologically almost exactly to the testicular portions of the hermaphroditic gonad and were thus used as a typical male control in the microscopic study.

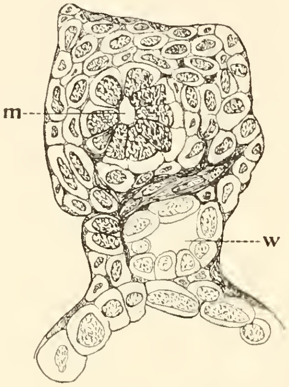

FIG. 3. Cross section showing (*m*) the Müllerian and (*w*) the Wolffian ducts in the adult hermaphrodite (cf. Fig. 1 *B*) (× 400).

The fat bodies in the hermaphrodite were especially large, and when the body cavity was first opened completely obscured everything beneath them, making their removal necessary for the study of the gonads. The right gonad is the larger and in a macroscopic examination seems to be primarily a testis with characteristic pigmentation and conspicuous lobules. The pigmentation is, however, somewhat lighter in color than that usually found in the adult testis. The length of the testicular portion of the right gonad is 4 mm., while that of the testis shown in Fig. 1 *A* is 5.25 mm. Posterior to this testicular portion, occurs a more slender unpigmented structure in which ten large unmistakable ova, together with smaller ones, may be seen. Its general resemblance to an ovary is seen by comparison with the ovaries of the 60 mm. adult female shown in Fig. 1 *C*.

The left gonad is longer and more slender as a whole than the right. This is due to the greater length of the ovarian part, the testicular region being smaller than that of the right gonad (2.75 mm. as compared with 4 mm.). Moreover the testicular pigmentation is confined to the anterior region of the gonad and is still lighter in color than that of the right gonad. The characteristic lobules are present, but there is less differentiation of the testicular from the ovarian region, the two seeming to grade into each other insensibly. In this gonad 14 large ova are in evidence as well as numerous smaller ones. At the extreme posterior end of the left gonad there is a small semi-detached ovarian lobe.

The hermaphrodite had not been preserved originally for histological study, since the animal had been killed in 5 per cent. formalin and had been kept in this fluid since 1915. Nevertheless the gonads were sectioned, and, in spite of the excessive shrinking which is especially evident in the separation of the cysts which make up the testicular lobules, the characteristic structure of both the male and the female components was shown with unmistakable clearness.

Figure 4 shows a cross section through a region where, in a macroscopic examination, the right gonad had the appearance of a testis and the left one the appearance of an ovary. In general this section shows the typical testicular structure of the right gonad, with lobules, each made up of a number of component cysts of male cells, arranged radially about a central collecting duct. A single large ovum appears in the section, however, completely filling one of the lobules and thus apparently the equivalent of many cysts. The characteristic anabolic and katabolic nature of the female and male cells respectively is thus well exemplified. The female cell grows, the male cell divides. In the whole series of sections through the gonads no fewer than ten such ova were found in the testicular portion of the right gonad and six in that of the left. All were, like the one shown in Fig. 4, in an apparently normal state of development, manifesting no incipient signs of degeneration such as Crew ('21) reports to be the case in Anura whenever

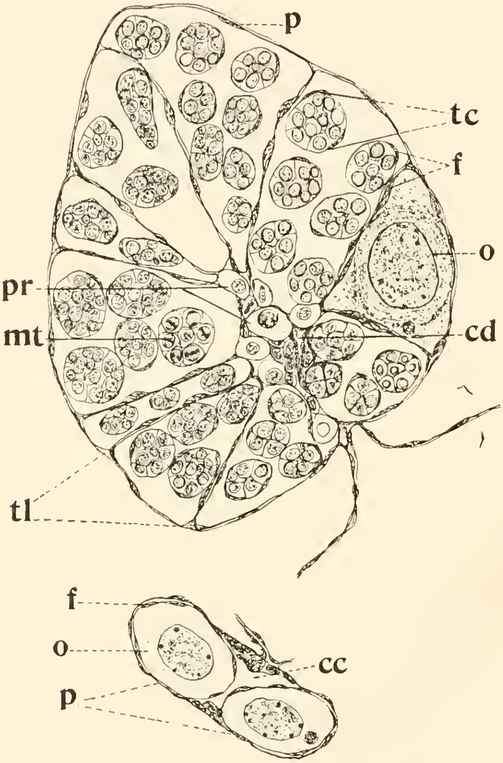

FIG. 4. Cross section through the gonads of the adult hermaphrodite at the level indicated in Fig. 1 *B* by the line 1–2 (× 87). The two gonads are brought nearer together in the drawing than their actual position. *Cc*, central cavity of ovarian region; *cd*, collecting duct of testicular region; *f*, follicles of both ova and testicular cysts; *mt*, spermatogonial cells in mitosis; *o*, ova (primary oöcytes); *p*, peritoneal investment; *pr*, primordial germ cells; *tc*, testicular cyst; *tl*, testicular lobule.

female elements are found in parts which are primarily male in character. In fact no difference could be detected between the ova among the testicular lobules and those of the more distinctly ovarian part of the gonads except that the former had advanced further in the matter of accumulation of layers of yolk.

There was much mitotic activity in progress in the testicular lobules, the same stage of mitosis being exhibited by all the cells of a given cyst, a condition which is to be expected if one postulates their formation by repeated divisions of a single primordial spermatogonium. Thus the male elements, like the female, have every appearance of undergoing perfectly normal development. In the transition region from the testicular to the ovarian part of the gonad, small testicular lobules appear which are somewhat degenerate in character.

The posterior part of each gonad shows the typical ovarian structure as demonstrated by the section of the left gonad in Fig. 4, with large central cavity surrounded by ova, each within its layer of follicle cells.

The microscopic condition thus shown seems scarcely more advanced than that pictured and described by Chapin ('15) in the gonads of her 46 mm. hermaphroditic "larva," which, in the absence of the more exact criteria of developmental stages of the whole larval life such as we are here making use of, was designated as a "larva" in the sense that it had not yet undergone transformation. In reality it was probably an individual which was approaching metamorphosis if not in actual metamorphic condition. In general this species shows much normal variation in the developmental condition of the gonads at transformation and it is thus not surprising that one individual previous to transformation should be in the same condition as another which has already transformed.

Our adult hermaphrodite is noteworthy, not only because it shows that the condition is not merely a juvenile one, but also because so far as external characters are concerned it appears to be a female. These characters, it must be confessed, are not of a very decided nature in this species, the presence of a spermatheca being, indeed, the only unquestionable one. Moreover, the cloacal papillæ which are the characteristic male structures, might not have appeared in so immature an individual and thus one cannot be sure that later the cloaca might not have shown male as well as female structures.

Crew ('21) in his summary of the recorded cases of abnormality of the reproductive system, says that of the 30 frogs of which sufficient details were given as to their secondary sexual characters, 25 (83.3 per cent.) were definitely and typically males; 4 others were definitely but imperfectly male (13.3 per cent.); and in the remaining case, a *Rana temporaria* described by Huxley ('20), the secondary sexual characters were female (3.3 per cent.). He says: "The abnormalities which have been recorded can be so tabulated that the first case most nearly approximates to the normal female and the last the typical male, with respect to the nature of both primary and secondary sexual characters. Thus arranged it is seen that the cases furnish an almost complete series of gradations which range from an individual almost completely female, to one almost completely male, and that the conditions found readily appear to be merely graded stages of a single process."

All of our other hermaphroditic examples of *Eurycea* were in too early a stage of development for secondary sexual characters to have appeared. However, so far as the condition of the gonads alone was concerned the same sort of graded series was found as that described by Crew in the frogs.

More thorough microscopic examination of gonads might, by disclosing occasional ova among the lobules of an otherwise normal testis or a few testicular elements concealed by the large ova of an ovary, yield a more complete seriation. At least the conditions shown by *Eurycea* indicate that in this species the hermaphroditic condition cannot be interpreted as always a modification of the same sex.

Metamorphic Stage.

In the more advanced developmental stages, as in the case of the adults, the sexing of the specimens consisted in distinguishing between a large unpigmented ovary full of bulging ova and a more slender, heavily pigmented testis with, of course, attention directed toward the detection of any combination of the two, which would mean an hermaphroditic condition. Fig. 5 *B* shows the hermaphroditic gonads of a 42.4 mm. animal in the advanced metamorphic stage. The gonads are essentially male so far as general shape and slight characteristic pigmentation are concerned, and there are visible in them ten unmistakable ova. Figs. 5 *A* and 5 *C* represent the gonads of a typical male and female collected on the same date and of approximately the same size and stage of development, which may be used for comparison. As noted before, the smaller size of the hermaphroditic gonad, and of the ova present in it, is obvious.

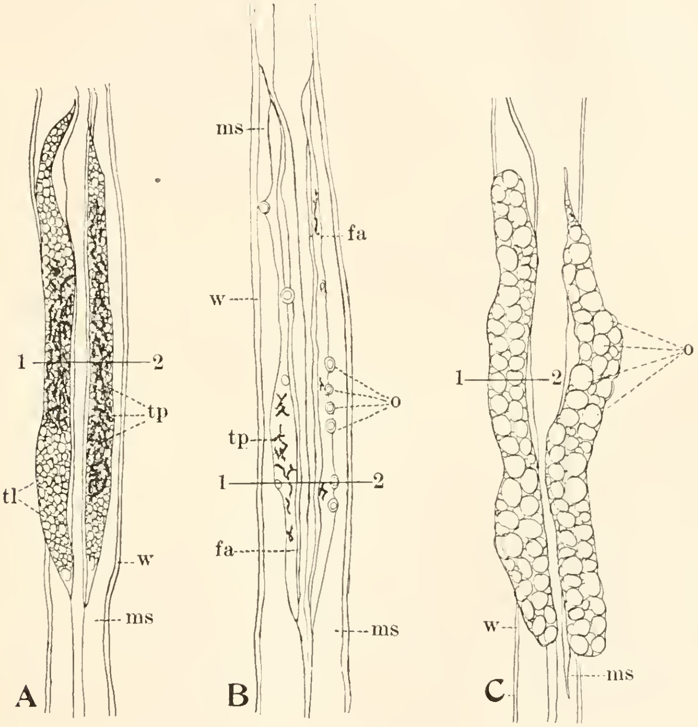

Fig. 5. Camera lucida drawings of the ventral view of gonads of (*A*) an incipient metamorphic male, length 40.9 mm.; (*B*) an advanced metamorphic hermaphrodite, length 42.4 mm.; and (*C*) a premetamorphic female, length 40.2 mm. (× 15). *Fa,* fat bodies; *ms,* mesonephros; *o,* ova; *tl,* testicular lobules; *tp,* testicular pigment; *w,* Wolffian duct. The levels of the sections of the gonads shown in Fig. 6 are indicated by the lines 1–2.

Cross sections 10 micra thick were made through all three pairs of gonads and were stained, some with Delafield hæmatoxylin,

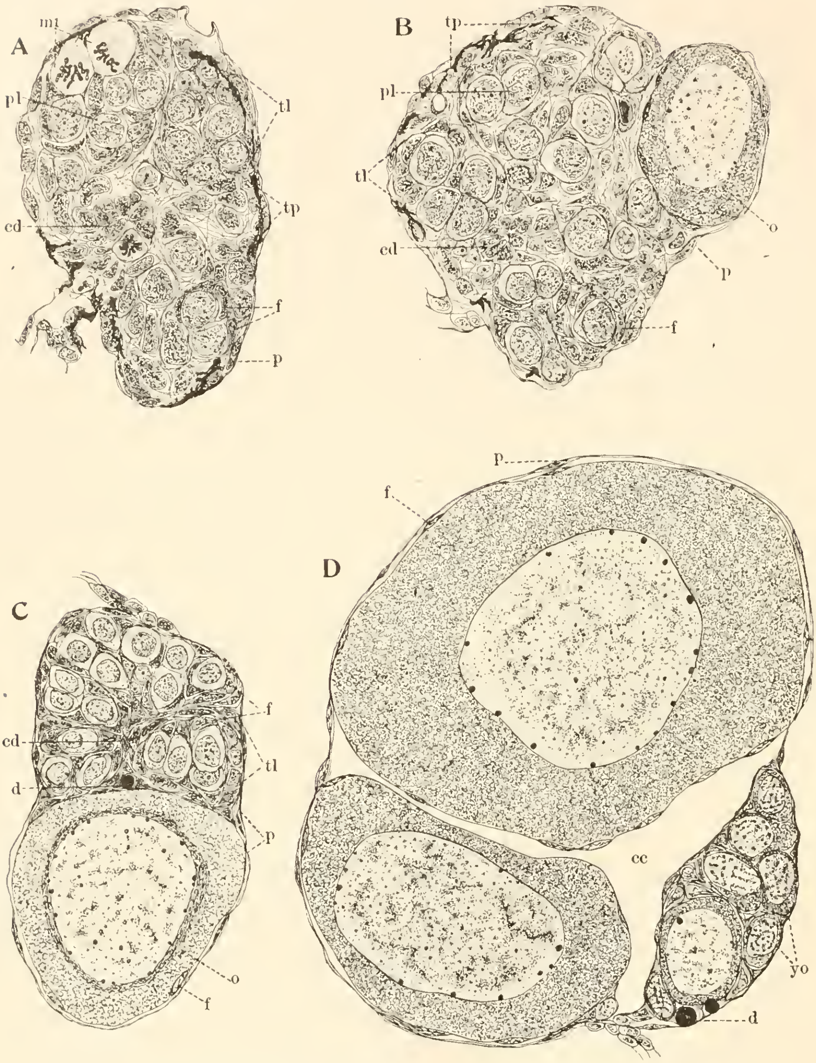

FIG. 6. Cross sections, at the levels indicated in Figure 5 by the lines 1–2, showing (*A*) the right gonad of an incipient metamorphic male; (*B*) the right gonad of an advanced metamorphic hermaphrodite; (*C*) the left gonad of the same individual; and (*D*) the right gonad of a premetamorphic female (× 365). *Cc,* central cavity of ovary; *cd,* collecting duct of the testis and testicular region of the hermaphroditic gonad; *d,* degenerating cells; *f,* follicles; *mt,* spermatogonial mitoses; *o,* ova (primary oöcytes); *p,* peritoneal investment; *pl,* polymorphonuclear germ cells (primary spermatogonia); *tl,* testicular lobule; *tp,* testicular pigmentation; *yo,* young primary oöcytes.

others with iron hæmatoxylin, and still others with safranin and light green. The lobules of the normal testis (Fig. 6 *A*) are made up of spermatogonia surrounded singly or in small groups with follicle cells, with which the cysts later to be formed by the division of these spermatogonia will be covered. Typical spermatogonial mitoses are seen in this and in other sections. In the hermaphroditic gonads (Figs. 6 *B* and *C*) we find a testicular structure corresponding in general to that shown by the normal testis, with typical mitoses in evidence. At the level shown in *C* in which the ovum constitutes practically half of the total diameter of the gonad, the testicular part is not quite so far advanced as in *B,* but is, in fact, in much the same condition as the more anterior region of the normal testis. The ova shown in both of these sections are typical, as will be seen by comparison with the section of the normal ovary (Fig. 6 *D*), although they are not equal in size to the larger ones of the normal ovary.

Incipient Premetamorphic Stage.

In the examination of younger stages in which little or no testicular pigment had developed, reliance for the diagnosis of the sex had to be based upon the shape of the ovary with its protruding ova to distinguish that organ from the slender testis or from the testis with female elements present in it.

Figure 7 *B* shows the general appearance of the hermaphroditic gonad of a 36 mm. incipient premetamorphic individual, and Figs. 7 *A* and *C* show gonads of a typical male and female of about the same size and developmental condition. The smaller size of the hermaphroditic gonad is again evidenced. The ova are of about the size of the smallest seen in the normal ovary.

The anterior part of the reproductive organs in each case was sectioned transversely for the purpose of studying the relation of the ducts; while the posterior part, including, in fact, the major part of the gonads themselves, was sectioned horizontally. Delafield hæmatoxylin and iron hæmatoxylin staining were used.

Histologically the developmental condition of these gonads as shown in Fig. 8 *A, B,* and *C* is not essentially different from that of the metamorphic stage, except that both in the normal testis and in the testicular region of the hermaphroditic gonad there are more single spermatogonia and fewer in groups, although the lobulated structure of the gonad is evident. There were fewer instances of spermatogonial mitosis, none, in fact, discovered in the hermaphrodite, the sections of which, however, were somewhat fragmentary. In every other particular of cell arangement and nuclear structure the testicular regions of the hermaphrodite were identical with the normal testis. The ova of the hermaphrodite,

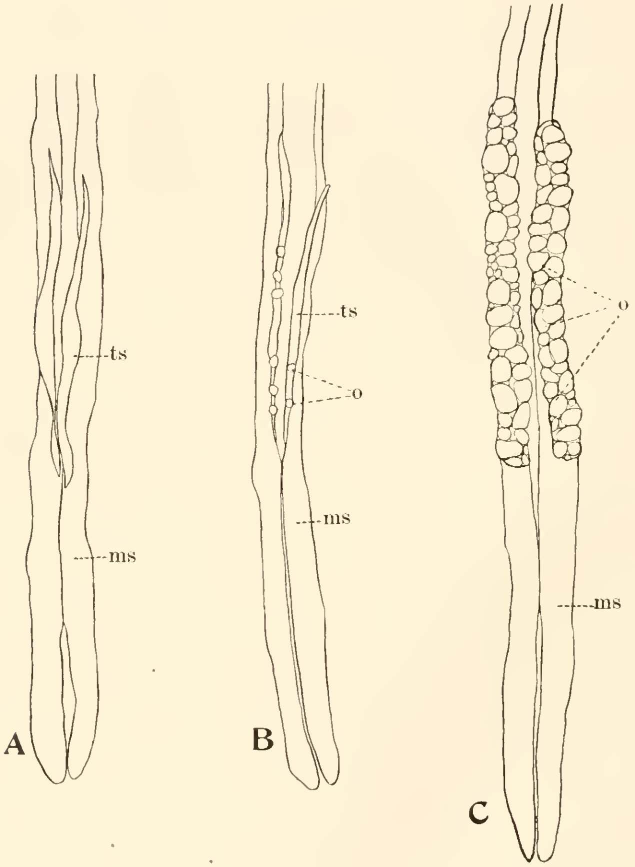

FIG. 7. Camera lucida drawings of the ventral view of the gonads of (*A*) an incipient premetamorphic male, length 37.4 mm.; (*B*) an incipient premetamorphic hermaphrodite, length 36 mm.; and (*C*) an incipient premetamorphic female, length 36.2 mm. (× 15). *Ms,* mesonephros. *o,* ova; *ts,* testis and testicular portion of hermaphroditic gonad.

though of smaller size, were perfectly normal in appearance. They have the typical relationship to the testicular lobules, and, owing to their earlier stage of growth, do not bulge out so conspicuously from the surface of the gonad as in the case of the metamorphic stage (cf. Fig. 6 *B* and *C*).

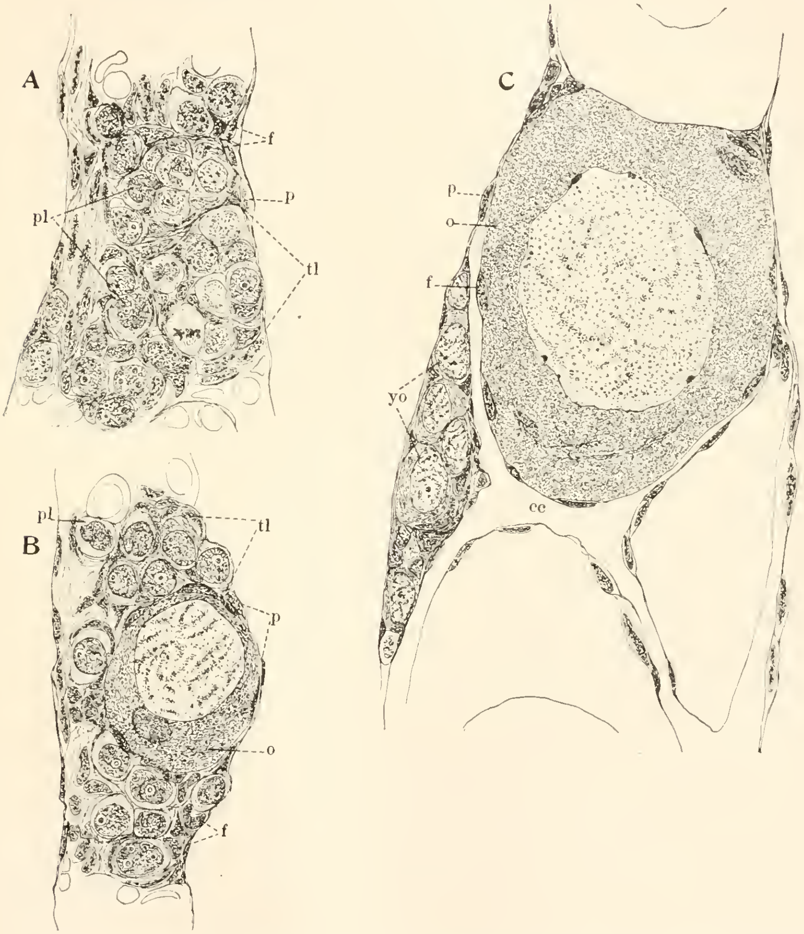

Fig. 8. Horizontal sections through corresponding regions of the gonads shown in Fig. 7 of (*A*) male; (*B*) hermaphrodite; (*C*) female (× 356). *Cc,* central cavity; *f,* follicles; *o,* ova (primary oöcytes); *p,* peritoneal investment; *pl,* polymorphonuclear germ cells (primary spermatogonia); *tl,* testicular lobule; *yo,* young primary oöcytes.

Typical Larval Stage.

With the younger stages, macroscopic evidence could be relied upon still less for sexing, although normal ovaries are easily recognizable if the growth period of the ova has been well entered upon, and such normal female gonads packed with growing ova of approximately uniform size have been distinguished macroscopically in larvæ as small as 25 mm. in length, though our percentage data (p. 8) did not include individuals under 27 mm. in length. The difficulty in sexing lies in the uncertainty as to the presence of male elements in gonads in which the ova are few in number but unmistakable. We have not as yet examined microscopically large numbers of gonads of young larvæ. However, in looking over our laboratory sets of serial sections of larvæ collected in September or early October, ranging in length from 17 to 25 mm. and presumably about 12 weeks old, we find that while a few of them show a condition which might be considered as sexually indifferent in that the gonads are made up of typical primordial germ cells, each with its investment of follicle cells, arranged in single rank about a central cord, a larger number of those examined, including some of the smallest individuals, show practically all of the germ cells in early maturation stages (leptonene and pachytene) or as growing oöcytes. Such an individual seems to us to be a female, since other individuals show gonads made up of more numerous, smaller germ cells grouped in such a manner as to suggest at once the incipient lobules of a typical testis. In such gonads the germ cells show no maturation phenomena, although mitosis is ocasionally seen. For the most part the nuclei are either polymorphic, or in rounded form with one or more conspicuous nucleoli. As we have very few data as to the condition of the gonads the following spring, we can only express here our tentative opinion that this species, in spite of its prolonged larval life, exhibits no such early larval maturation of male germ cells synchronously with that of the female germ cells, as has been described by Swingle ('21) for *Rana catesbeiana*.

To push our power of diagnosis of sex and recognition of possible hermaphrodites back into these early stages demands as a basis, not only a careful and thorough cytological investigation of the origin and differentiation of the germ cells such as that of

Bouin ('01) and Dustin ('07) for other species of Amphibia (mainly *Anura*), and Okkelberg ('21) for the brook lamprey, but also a complete bridging over of the gap between the early developmental phenomena and the seasonal sexual phenomena of adult life.

With regard to the bearing which occasional hermaphroditism such as this has upon its regular occurrence in certain species of animals, and upon the significance of the phenomenon in general, two opposing views are held. One of these, as set forth by Doncaster ('14), regards hermaphroditism not as a primitive but as a purely secondary condition. This opinion is based mainly on the fact that the hermaphroditic species of animals are, for the most part, highly specialized ones. Sporadic hermaphroditism is thus considered an example of variation along this same direction.

The other view is that which has recently found so vigorous a supporter in Jordan ('22), that hermaphroditism, at least in the vertebrate group, is a primitive character. Jordan points out "the abundant evidence of a normal hermaphroditic condition either adult or juvenile, among lower vertebrates (*e.g.,* tunicates, cyclostomes, probably some Amphibia)," and that "the early gonads with their primordial germ cells appear identical." This view of the primitive character of hermaphroditism naturally goes hand in hand with the theory that sex determination is a matter of differential metabolism and that forms in which sex determination has become bound up in the chromosomes represent a higher stage in metabolic control of the developing organism.

Jordan points out the peculiar interest presented by the case of amphibians in this connection, since most investigators have failed to find any evidence of a sex chromosome in this group, although King ('12) describes it for *Necturus maculatus,* Levy ('15) for *Rana esculenta,* and Swingle ('17) for *Rana pipiens.* In a later paper, however, Swingle ('21) questions the correctness of his own earlier identification of an accessory chromosome in *Rana pipiens* and suggests the strong probability that Levy may also have been mistaken.

Jordan makes the suggestion that the Amphibia may constitute a group in which the evolution of the sex chromosome as a separate element can be traced, and in which also a general tendency toward juvenile hermaphroditism bridges the gap between lower vertebrates where functional hermaphroditism occurs in certain classes and higher vertebrates where the condition occurs only as an anomaly. Swingle has done much to dispel the idea of juvenile hermaphroditism in the anurans. Cases of hermaphroditism such as we have here described in *Eurycea bislineata* give every evidence of being a permanent rather than juvenile condition. On the other hand, although we have thus far found no evidence that these permanent hermaphrodites arise out of an earlier condition in which the gonads have the potentiality for both sexes and may thus be regarded as capable of producing either males, females, or hermaphrodites, we do not feel that our investigation of these early stages has been sufficiently extensive to warrant us in excluding this possibility. In the absence of evidence of a chromosomal control of sex determination in this species, and indeed in Amphibia in general, one should maintain an open mind toward other possibilities. Much further investigation of the subject is therefore obviously needed and is now in progress.

SUMMARY.

1. True hermaphroditism occurs in approximately constant proportions in every developmental stage of *Eurycea bislineata* from typical larval to adult.

2. The percentage of occurrence of hermaphrodites in this species, based upon the examination of 1,113 individuals, is 1.35 per cent.

3. There are now on record 18 cases of hermaphroditism in urodeles. The first is that of La Valette St. George ('95) in *Triton tæniatus,* the second is that discovered by Chapin ('15) in *Eurycea bislineata,* the third is that of Křizenecky ('17) in *Triton cristatus,* and the other 15 cases, in *Eurycea bislineata,* are presented in this paper.

DEPARTMENT OF ZOOLOGY, SMITH COLLEGE,
NORTHAMPTON, MASS.
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